JOHNSON, BROOKE A., M.S. The Lettuce Head Cells of the SCANS Region of the *Drosophila* Larval Midgut are Required for Larval Midgut Peristalsis. (2008) Directed by Dr. Dennis LaJeunesse. 73 pp.

The alimentary canal of the *Drosophila* larva is essential for survival of the organism. The gut tube is an intricate network of tissues interacting with the parasympathetic nervous system. The midgut, the region of the *Drosophila* gut that has the largest variety of cell types, plays a vital role in digestion and has been minimally studied. We have discovered a novel region in the larval midgut that we believe to be a portion of the *Drosophila* autonomic nervous system, we call this region the SCANS, or Superior Cuprophilic Autonomic Nervous System. This region has three distinct features: 1) 7-9 novel enteroendocrine cells, 2) a valve-like portion, and 3) muscular attachments to the dorsal gastric caeca. The novel enteroendocrine cells have a unique bottle-shaped structure with an apical lamellipodial head that projects into the lumen, from which they get their name the lettuce head cells (LHC), and a large body that is embedded within the endothelial lining of the midgut. The posterior projection and attachment of the dorsal gastric caeca to the SCANS region have allowed us to rearrange the anatomical design of the *Drosophila* larval midgut from the previous dogma wherein all four caeca extend anteriorly. The discovery of the SCANS region of the midgut, and the LHC, has led us to believe that there is peristaltic regulation occurring much like regulatory cells of the human digestive system. Given the location of the LHC in the gut tube, the expression of nervous system markers such as *elav, Ddc* and *Cha* in LHC, and the association of LHC with longitudinal visceral muscles, I will present in this study that the LHC are required for the regulation of muscle contractions in the larval midgut. I have effectively screened 37 *Gal 4* enhancer traps and recorded which ones are expressed both in the midgut and specifically in the LHC alone. One of which, *DJ752*, expresses solely in the LHC during third instar of larval development, and was chosen as the experimental line for many of the

assays. I have effectively cloned and characterized the *DJ752 Gal 4* insert, which is located within the *Enhancer of Split* [*E(spl)*] region on chromosome 3, and with this information have concluded that the LHC are indeed enteroendocrine cells. Ablation of the LHC using cytotoxins with the *Gal 4/Gal 80^{ts}* system resulted in total loss of peristaltic movement within the larval midgut. Neither the morphology nor the digestive ability of the larvae were compromised when the LHC were ablated. Ectopic activation of the LHC using the *Channelrhodopsin-2* transgene resulted in a significant increase in gut contractions showing that the anterior midgut functions as an independent unit from the remainder of the *Drosophila* alimentary canal.

THE LETTUCE HEAD CELLS OF THE SCANS REGION OF THE *DROSOPHILA* MIDGUT ARE REQUIRED FOR LARVAL MIDGUT PERISTALISIS

By

Brooke A. Johnson

A thesis submitted to
the Faculty of The Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirement for the Degree
Master of Science

Greensboro
2008

Approved By

_____
Committee Chair

In loving memory of those influential, strong, and unforgettable people who have had to leave this world during my life. To my Grandmother, to Paul, and to Nancy - I promise to continue on my path, to never look back, and to live life as if every day were my last.

APPROVAL PAGE

This thesis has been approved by the following committee of the faculty of The Graduate School at The University of North Carolina at Greensboro.

Committee Chair: _____
Dr. Dennis LaJeunesse

Committee Members: _____
Dr. Mark Hens

_____
Dr. Karen Katula

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEGEMENTS

I would like to thank Dr. LaJeunesse for his endless guidance, support, patience, and sense of humor that he has contributed to my success in completing this project. His endless wealth of knowledge, innovative ideas, and understanding were all instrumental to my ability to take this step forward in my scientific career. He is an incredible mentor and teacher and has given me a foundation that will aid me in all walks of life. I will be forever grateful for the wisdom and priceless memories I have been given and will never forget my experience here.

I would also like to thank Dr. Katula and Dr. Hens for being such influential teachers and mentors in my journey here at UNCG. Their input into my work has been extremely valuable, their understanding of life situations has been wonderful, and their guidance has really helped me bloom into the person I am today. Additional thanks to Dr. Adamson for her patience and input that was desperately needed throughout my project.

Finally, I would like to thank Nate and my family. Without you to keep me strong in trying times I would not have continued on this path to pursing my dreams. You are my rock, my foundation, and without you none of this would have been possible.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

The Digestive System

The acquisition of nutrition from the environment is central to life and all higher metazoans have evolved digestive systems that extract food from the environment for energy. Although digestive systems have evolved to adapt to diverse feeding behavior, they share an overall similarity in their organization and development. Most alimentary canals are tubes located within the body and most of the digestive system is derived from endoderm (the tube) and mesoderm (the muscles). Often, the genes that control these digestive processes are well conserved evolutionarily (Ohlstein and Spradling, 2006). Interestingly, but not surprisingly, the acquisition of nutrition involves a coordination of the nervous and endocrine systems to govern the rate of feeding and the processing of food within the alimentary canal (Chaudhri et al., 2006). For instance, feeding is guided by neuronal sensory input that guides the animal to food sources and promotes active feeding, while hormonal signals activate metabolism, stimulate hunger, and increase the amount of food intake (Benoit and Tracey, 2008; Valassi et al., 2008; Chaudhri et al., 2006). Once digestion begins, other internal neural sensors within the gut proper begin to regulate the rate of digestion as similar hormones stimulate the cells of the digestive tract to begin functioning in specialized ways (Chaudhri et al., 2006).

The study of the controls of digestion has been best studied in vertebrate systems. The hormonal component of feeding and digestion is well described in the studies of such hormones as leptin, neuropeptide-Y, insulin, cholecystokinin (CCK), and melanin-concentrating hormone (Benoit and Tracey, 2008; Valassi et al., 2008; Chaudhri et al., 2006). Furthermore, in vertebrate intestines, digestion and the movement of food are regulated by the two components of the autonomic nervous system; the parasympathetic nervous system (PNS), which innervates the visceral smooth muscles of the guts, and the enteric nervous system (ENS), which is a network of neuron-like cells responsible for governing gut movements, and reside within the lower intestine (Meyer and Quenzer, 2005). Unlike the enteric ganglion, the cell bodies of the PNS are located in the ventral horns of the spinal cord; cranial nerves III, VII, IX, and X, and the sacral region of the spinal cord (Meyer and Quenzer, 2005). Primary ganglia, extending from the spinal cord level towards the digestive organ, secrete the neurotransmitter acetylcholine (Ach) and travel long distances where they synapse on short post-ganglia that directly innervate the intestine (Meyer and Quenzer, 2005). The neurotransmitter Ach is essential at the neuromuscular junctions to stimulate contractions of gut muscles to induce peristaltic movement, and is the neurotransmitter responsible for visceral muscular contraction (Meyer and Quenzer, 2005).

Intestines of higher metazoans have evolved to include the structure of longitudinal and circular smooth muscles that maintain the integrity and the tensile strength of the gut tissue (Park, 2000). The 'peristaltic reflex,' provided by the ENS, is responsible for most propulsive motility patterns in the bowel. Stimulation of the

intestinal wall, or mucosa therein, will result in a systematic "reflex" where the circular muscle coat will relax at the point of stimulation, the longitudinal muscles will contract below the point of stimulation, as well as contraction of the circular muscles above the stimulus (Wood, 2008). The cell body of the enteric ganglion is located in the dorsal horn of the spinal cord where internuncial centers consisting of interneurons will modulate signals to and from sensory neurons to the afferent limb, and from efferent limbs, to motor neurons and their effector organ, allowing the reflex arc to function separately from the CNS. The enteric ganglion is often referred to as the "gut-brain," because of its ability to act as an independent nervous system due to the low level of complexity involved in the 'peristaltic reflex' (Wood, 2008).

In addition to the parasympathetic nervous system input and the enteric ganglion, other neural-like cells contribute to muscular contractions within the vertebrate intestine. Over 100 years ago, Ramon Y. Cajal described a set of nerve-like cells in organs innervated by peripheral nerves (Sanders and Ward, 2006). These cells are found throughout the human body in numerous tissues such as the heart and bladder and are best described in the smooth muscle of the gastrointestinal tract (Sanders and Ward, 2006). Initially these cells were first identified via the histochemical staining techniques that scientists used to identify neurons, however their structure and organization remained vague because of the imaging limitations of this time. With the advent of electron microscopy these Interstitial Cells of Cajal (ICC) were shown to be in close apposition with nerve terminals and form gap junctions with smooth muscle cells of the intestine (Sanders and Ward, 2006). In mouse embryos, removal of the ICC by blocking the Kit

pathway resulted in a reduction of neural responses and the abolishment of pacemaker activity throughout the GI tract (Sanders and Ward, 2006).

There are two populations of ICC in the GI tract. One is between or at the edges of muscle layers that generates and propagates electrical slow wave activity and the other is within muscle bundles in close apposition with enteric neurons (Sanders and Ward, 2006). ICC generate rhythmic oscillations, known as unitary potentials that can summate in response to depolarizations and in fact, regulate the dominant pacemaker cells. The myenteric plexus of the ENS, located between the two layers of visceral muscle outside the intestine, makes up the dominant pacemaker region of the gut. The frequency of pacemaker activity can also be regulated by the ICC acting as stretch receptors influencing the membrane potential (Sanders and Ward, 2006).

The movement of food through the alimentary canal requires the autonomic nervous system and many human aliments, including Hirschsprung's disease and Gaucher's syndrome, result in the cessation of digestion, or the movement of food along the alimentary canal (Amiel J. et al., 2007). The ICC are vital to the motility of the GI tract, as many motility disorders occur because these cells have ceased to exist or stopped functioning. These include such disorders as: pseudo-obstruction, gastroparesis, pyloric stenosus, and lower oesophageal sphincter achalasia (Sanders and Ward, 2006).

Other non-neural cell types exist in the human digestive system that effect digestion. There are four main cell types in the intestine: absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells (Cheng and Leblond, 1974; Evers 1999). Among the more interesting and important enteroendocrine cell types are L-cells, which

are located in the human duodenum and secrete glucagon-like peptide-1 (GLP-1) in response to the presence of glucose (Jang et al., 2007). These cells, much like the taste cells of our tongue, actually "taste the sweetness of glucose" through the G protein gusductin and other taste transduction elements (Jang et al., 2007). The release of GLP-1 as a result of "tasting glucose" in the digestive tract, allows for regulation of appetite, insulin secretion, and gut motility (Jang et al., 2007).

Overview of the Drosophila larval gut

The organization of the Drosophila larval gut is far simpler than its vertebrate counterparts, however it still contains all of features of a complete alimentary system such as an endothelial tube, visceral muscles, and interacting portions of the nervous system (Klapper 2000; Nagagoshi 2005). This simplicity will allow us to use the larval midgut as a model to study digestion; however, this system in *Drosophila melanogaster* is woefully understudied. While the general organization of the larval and adult *Drosophila* alimentary canals has been described, little work on specific details within the gut and the mechanisms that control and regulate digestion and the movement of food has been done. With its rapid lifespan, enormous collection of genetic and cell biological tools, and simple digestive system, *Drosophila melanogaster* provides an excellent system to study these mechanisms at the cellular and genetic level.

The *Drosophila* larval midgut consists of a two thin layers of mesodermally derived visceral muscle that sheath an endodermally derived endothelial tube (Nagagoshi, 2005). The endothelial tube consists primarily (90%) of large absorptive enterocytes and

to a lesser extent various types as of yet undescribed enteroendocrine cells (10%). These two general cell type arise from stem cell crypts and differentiate using Notch signaling (Michellli and Perrimon, 2006; Ohlestien and Spradling, 2006). Specification of either cell type in the endothelial tube is dependent on positional values and signals that still remain unclear. However, possible signals from the Wnt and dpp pathways, as well as the homeotic genes probably play key roles (Nagagoshi et al., 2005).

The alimentary canal of *Drosophila* is segregated into three major parts: the foregut, midgut, and hindgut, all of which arise from the anterior and posterior invaginations of the early blastoderm (Murakami et al, 1999) (Figure 1). Ultimately, the developing midgut undergoes a distinct regional differentiation that leads to the unique appearance of the larval midgut. The large central sac contained within the fused midgut rudiments begins to elongate into a tube by the formation of a number of constrictions and indentations. A constriction anterior to the level of the middle midgut rudiment forms separating the most anterior portion of the anterior midgut rudiment from the body of the midgut. This region becomes the proventriculus, or cardia (Demerec, 1965). Two more constrictions develop within the midgut below the first anterior constriction as well. There is a constriction at the level of the middle midgut rudiment equally spaced from the first, and a constriction at the posterior-most part of the midgut, where the Malpigian tubes will develop (Demerec, 1965) (Figure 1). The middle midgut region begins as a slender area that eventually expands into a larger stomach region that is typically filled by food. The anterior end of this region develops four diverticula, or blind sacs, called the gastric caeca (Figure 1). Two of these caeca are paired and disposed dorsally, while two

more push out ventrally (Demerec, 1965). In all previous literature the caeca are said to extend cephallly and their cell morphology is consistent with the rest of the cells in this stomach-like region (Demerec, 1965). The posterior tapering, which falls below this stomach-like region, marks the site at which the pH of food is neutralized. Following this constriction, the longest portion of the midgut exists that is most likely the principal site of food absorption (Dubreuil, 2003). This portion of the midgut is segregated into three basic regions where the epithelial cells specific to these caudal portions of the midgut perform the function of reabsorption (Nagagoshi et al., 2005; Demerec, 1965) (Figure 1).

The final result of this differentiation process is an elaborate section of the larval intestine designed for specific and vital gut functions. The midgut epithelium, or endothelial epithelium, in total, is described as a single layer of large digestive cells with small regenerative cells between the bases of the larger cells attached to the basement membrane. Furthermore, within the midgut tube itself there are subtle but real differences within the type and organization of endothelial epithelium (Demerec, 1965). For instance, cells making up the wall of the proventriculus have been described as compacted and columnar, whereas in other portions of the gut, such as the gastric caeca, endothelial cells express a more cuboidal cell type (Demerec, 1965). The central midgut also has specialized cells that were first described by Strausberger in 1932 as "cuprophilic" cells (Dubreuil et al., 1998). Further research to date shows that these cells are responsible for acid secretion and have a function much like mammalian gastric parietal cells (Dubreuil et al., 1998). These cuprophilic cells have a unique structure much suited to their function, with a deeply invaginated apical membrane covered in

microvilli that regulates acidification of the stomach by opening and closing (Dubreuil, 2003). These "copper" cells are flattened, spherical shaped cells that were found to alternate with spindle-shaped interstitial cells, yet another specialized cell type in the *Drosophila* midgut (Dubreuil, 2003).

The visceral musculature of the *Drosophila* midgut is composed of two sets of striated muscles most similar in structure to cardiac striated muscle (Goldstein and Burdette, 1971). And in fact, the visceral muscles originate from the same group of dorsal mesodermal cells that give rise to the dorsal vessel, the *Drosophila* heart (Furlong , 2004). Much like the organization of vertebrate intestines, the visceral muscles of the *Drosophila* midgut are organized into two distinct groups: an inner group of circular muscles that wrap the circumference of the gut and 18-22 longitudinal muscles that traverse the length of the midgut (Klapper *et al*., 2002). The *Drosophila* visceral muscles are among the longest living cells in the fly with both the longitudinal and circular muscles persisting through metamorphosis (Klapper *et al*., 2002). Recent genetic studies suggest that the development, specification and differentiation of vertebrate cardiac and insect visceral muscle cells are evolutionarily conserved (Baylies et al., 1998; Broihier et al., 1998; Ranganayakulu et al., 1998; Kusch and Reuter, 1999). It is possible that the pacemaker system employed in the *Drosophila* visceral muscle is a primitive form of the pacemaker cells seen in the human heart and intestines. If a homology exists such as this, the *Drosophila melanogaster* may be yet another easily obtainable, simplistic model to study human cardiac muscle, the human digestive tract, and the autonomic nervous system.

Autonomic nervous system in the larval *Drosophila melanogaster* midgut

Both the foregut and the hindgut are innervated by nerves emanating from the peripheral nervous system (Demerec, 1965). However, only the proventricular/ anterior portion of the larval midgut is innervated by the CNS (Figure 1) and the mechanism that maintains and propagates peristaltic muscle action through the remainder of the larval midgut is unknown (Budnik et al., 1989; personal observation Dennis LaJeunesse). Although, the mechanisms of digestion are well studied in vertebrate systems, little is known about the ANS in invertebrate systems (Seroud et al., 2002).

Specific Aims

The purpose of my thesis research is to determine if there are novel areas in the *Drosophila* larval midgut that may lead us to a better understanding of the mechanisms involved in midgut innervation and peristalsis. Specifically, the goal of my project is to see what is required for muscle contraction and the movement of food, and to characterize this requirement. To do this I propose three specific aims.

- **Specific Aim I. Screen 37 *Gal4* enhancer trap lines to look for novel areas of the midgut and genes that express in the midgut.**

- **Specific Aim II. Clone a new gene expressed in the SCANS region in order to characterize the functions or development of this region.**

- **Specific Aim III. Characterize the functional requirements of the LHC in midgut peristalsis.**

. To determine the requirement of the LHC for midgut peristalsis I will employ two genetic techniques to disrupt normal function of the LHC in the SCANS region.

i. LHC Ablation using *UASricin/UAS reaper/Gal4/Gal80^{ts}* system.

ii. Ectopic activation of LHC using *UASChR2* expression.

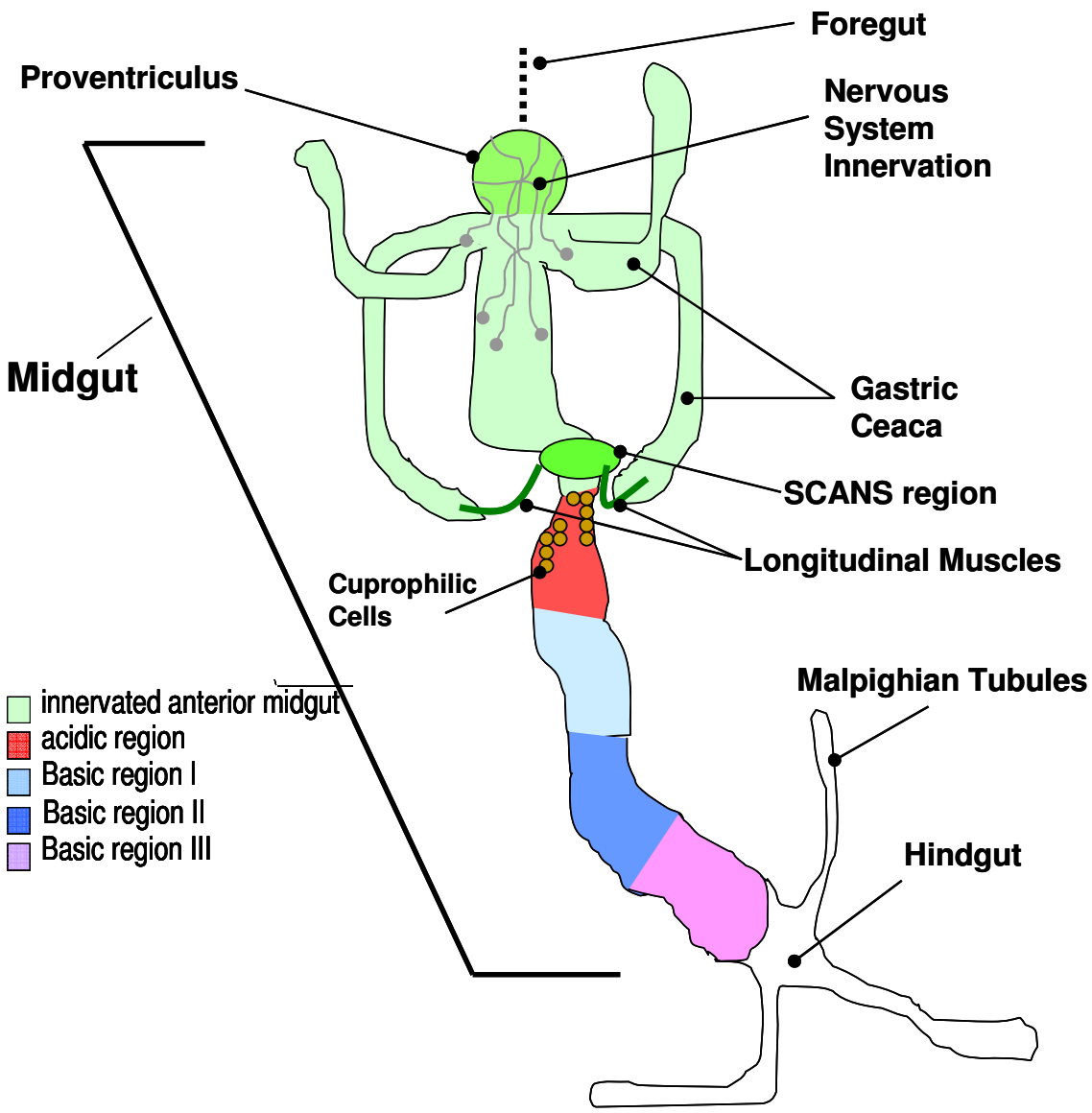


Figure 1: *Drosophila* alimentary canal.

CHAPTER II

MATERIALS AND METHODS

Drosophila **Genetic Strains**

All *Drosophila melanogaster* were raised in plastic vials on a medium of standard cornmeal, yeast, molasses, and agar with methyl-4-hydroxybenzoate added as a mold inhibitor. The stocks remained at room temperature 22°C unless otherwise noted. The stocks used in this study are as follows: w^{1118}, which was used as the wild-type strain; the following *Gal 4* enhancer trap lines from the Bloomington Stock Center, Bloomington Indiana: *MJ33a, MJ12, DJc564, DJ752, DJ717, CD20, CD30, C805, ChaGal4, BabGal4, DdcGal4,*. The following *UAS GFP* stocks were used for the characterization of staining patterns of the *Gal 4* enhancer traps and the LHC: *UAS::CD8GFP*, *UAS::actin GFP*, *UAS::mitoGFP*, *UAS::gRASp GFP* (golgi marker), *UAS::TubulinGFP*. We used the following stocks for the cell ablation experiments: *UAS::ricin* (courtesy of K.G. Moffat, University of Warwick), *UAS::rpr.c* (Bloomington Stock Center, Bloomington Indiana); and *Ubi::Gal80*^{ts}(Bloomington Indiana). For the gain of function experiments *UAS::ChR2 II + III* (courtesy of Andre Fiala, Department of Genetics and Neurobiology, Theodor-Boveri-Institut, Julius-Maximilians-Universität Würzburg).

Quick Genomic DNA extraction protocol for Plasmid Rescue

30 anesthetized *DJ752 Gal4* flies were collected and frozen at -80°C. Following freezing, flies were ground into 200uL of Buffer A solution (100mM TrisHcl; 100mM EDTA; 100mM NaCl; .5% SDS) until only the cuticles of the flies remained. An additional 200uL of Buffer A solution was added and the mixture was incubated for a period of 30 minutes at 65°C. A solution of LiCl/KAc (1 part 5M KAc stock : 2.5 parts 6M LiCl stock) solution was added and the mixture was incubated on ice for a period of 10 minutes. Following the second incubation, the mixture centrifuged at 14000 rpm, room temperature, and the supernatant was transferred into a new microtube avoiding any floating material. 600uL of isopropanol was mixed into the supernatant, and again the mixture was centrifuged at room temperature for 15 minutes. The supernatant was discarded and the pellet was washed using 1mL of a 70% ethanol solution for approximately 1 minute. The DNA pellet that remained was resuspended in 100uL of TE and stored at -20°C overnight.

Plasmid Rescue Protocol

The plasmid rescue protocol was used to clone DNA flanking the *DJ752* insert is summarized in Figure 2. Genomic DNA suspended in TE was digested with several different restriction enzymes in separate reactions: *XhoI*, *EcoRI*, *SalI*, *BGLII*, *PstI*, and their associated buffer solutions. The digestion mixture consisted of: 15uL of deionized H_2O, 2uL of the genomic DNA (between 200 and 500 ug), 2uL of 10xBuffer, and 1uL (2

units) of the appropriate enzyme. The mixtures were immediately transferred to a water-bath and incubated for 2.5 hours at 37°C. After the incubation period, the digestion mixtures were transferred to a dry bath incubator and were maintained at 65°C for 20 minutes. 10uL of each digest was run on a 0.8% agarose gel along side uncut DNA fragments to ensure that the DNA from the digestion was cut by the restriction enzyme in question.

10uL of the digested genomic DNAs were then combined with 350uL of deionized H_2O in microfuge tubes. 40uL of 10X Ligation buffer was then added to each tube followed by NEB T4 DNA ligase (1 unit). There were 5 separate ligation reactions for each enzyme used. The ligation mixture was then incubated overnight at 4°C. After incubation, the mixtures were thawed on ice and the DNA was precipitated by washing the pellet with ethanol. The pellet was then dried and then re-suspended in 150uL of TE. The ligation was done in order to allow circular DNA fragments to form (Figure 2, d.).

The ligated/ circularized DNA was transformed into chemically competent *E.Coli* bacterial cell of the strain XL10 Gold (transformation efficiency of $1x10^7$ cfu/ug). 50uL of the cells were added to the ligated DNA samples and incubated on ice for 30 minutes. The microtubes containing the genomic DNA/competent mixture was transferred to a water-bath at 37°C for two minutes. The competent cells were then transferred to 1mL of LB media in a Falcon tube and incubated in a C2 incubator shaker at 37°C for 1 hour. This incubation period allows for the cells to heal and begin replicating the newly inserted DNA plasmid. 100uL of the transformation mix were plated on LB and ampicillin plates and grown overnight at 37°C.

The appearance of colonies indicated the presence of a circularized plasmid with the ampicillin resistance gene included in the genomic DNA flanking the *DJ752 Gal 4* insertion. The colonies were selected from the plates and grown up in overnight cultures in 3ml of LB/ampicillin. The plasmid DNA was extracted using a standard alkaline mini-prep protocol and sent off to MWG to be sequenced. Four independent colonies were selected to ensure that the rescued plasmid represented the same insertional event. The resulting sequence was analyzed by Blast analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine its genomic location.

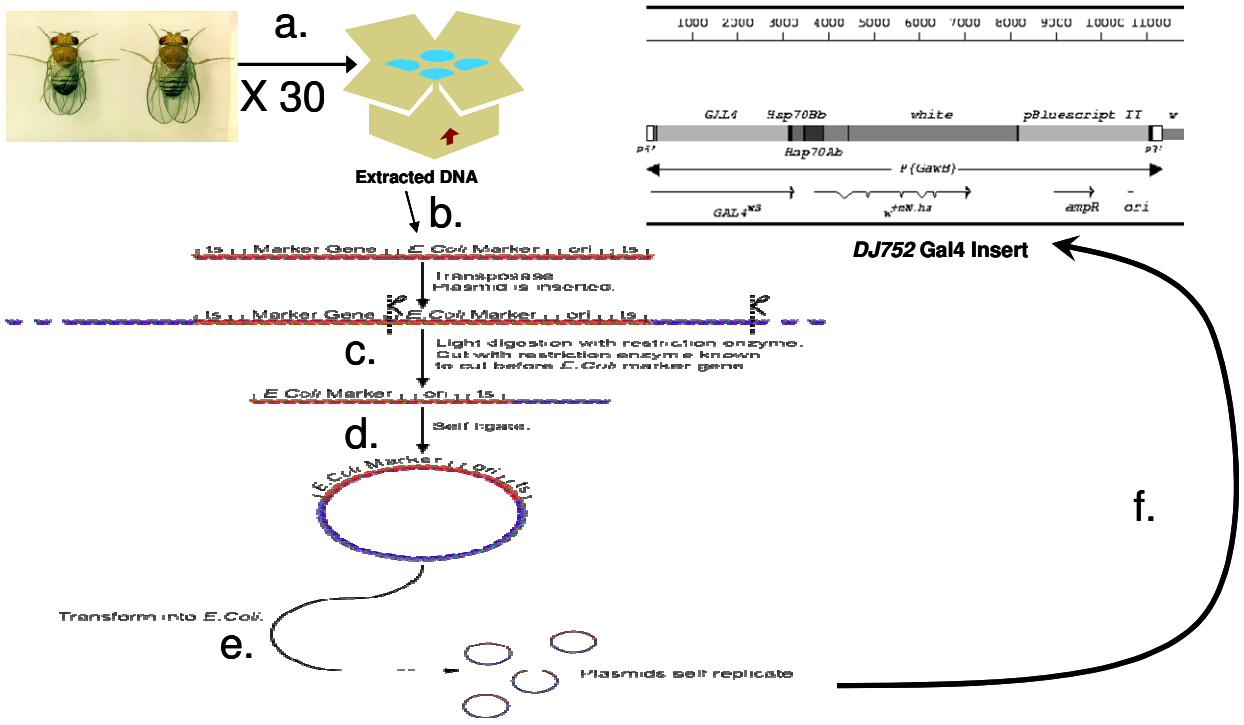


Figure 2: Plasmid rescue technique (Brand, 1993; www.flybase.org). a. ~30 flies were homogenized. **b.** DNA extracted using quick fly prep protocol. **c.** Digestion with *EcoRI* to cut *E. coli* marker gene which includes the insert in question. **d.** Ligation of newly cut DNA into plasmid form. **e.** Plasmids were transformed into the XL10-Gold *E. coli* cell line and incubated to allow replication. **f.** Alkaline analysis mini-prep was used to prepare plasmids for sequencing, and the *DJ752* plasmid was sequenced by MWG.

LHC Ablation using *UASricin/UAS reaper/Gal4/Gal80^{ts}* system

The following genetic crosses were set up to prepare fly lines for the LHC ablation. Virgin female flies with the genotype *w;UASricin/CyOGFP; Gal80^{ts}* were crossed with male flies with the genotype *DJ752UAS::CD8GFP* and *ChaGal4UAS::CD8GFP* (Figure 3). Female flies carrying *w;UASrpr.c/CyOGFP; Gal80^{ts}* were also crossed with both *DJ752UAS::CD8GFP* and *ChaGal4UAS::CD8GFP* (Figure 3). *w^{1118}* male flies were also crossed with the female flies carrying the cytotoxins *ricin* and *rpr.c* and were used as a control. The flies were maintained on standard cornmeal in vials and transferred daily to ensure optimal egg laying and to avoid overcrowding. Larvae were collected on the fifth day following egg laying. The larvae were separated from the cornmeal by mixing water in the vial and transferring the mix to a larger container, in which more water was added. The larvae were allowed to settle to the bottom of the container, and were flushed again with water. Larvae were then transferred to a smaller dish and visualized under a fluorescent microscope, where the non-glowing ones were picked out of the group. 20 to 30 non-glowing larvae were then placed in even smaller dishes containing a gel-agarose food dyed with 0.4 % bromophenol blue. These food dishes were then placed in an incubator overnight at 29°C and during this time period the cells now expressing the cytotoxin will die. Approximately 8 hours later, the dishes were removed from incubation, and allowed to stand at room temperature for 3 hours.

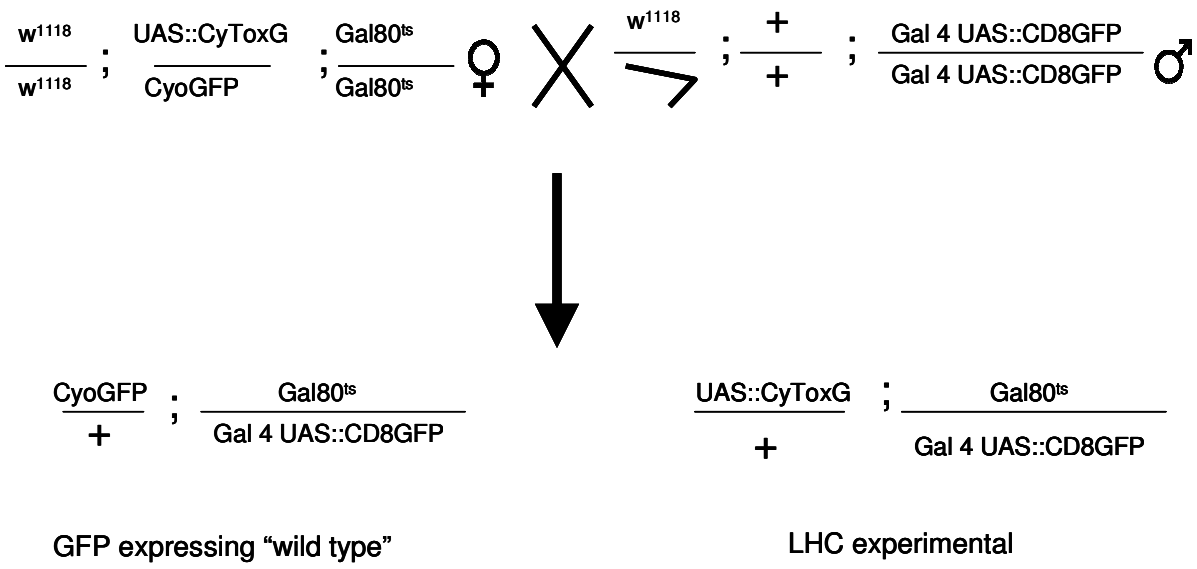


Figure 3: Map of genetic crosses for ablation experiments. One of two cytotoxins were used for the female flies, *ricin* or *rpr.c*. Either *DJ752* or *Cha Gal4* driver lines were used for male flies. Offspring of this cross resulted in either GFP expressing "wild type" flies with the *CyO* balancer, or the experimental flies. Experimental flies employ the *Gal4/Gal80^{ts}* driver system where both the cytotoxin and GFP expression will be suppressed by *Gal80^{ts}* at room temperature. Flies were placed under a fluorescent blue light and segregated according to their GFP expression at room temperature. Non-glowing, or experimental, larvae were placed at 29°C overnight to block *Gal80^{ts}* and activate the expression of the cytotoxin and GFP *Gal 4* expression simultaneously.

Feeding Assay

Larvae of the following genotypes were reared on the standard medium at room temperature until third-instar (5days): w^{1118} , *w;UASrpr.c/CyOGFP; Gal80^{ts} x DJ752UAS::CD8GFP*, *w;UASricin/CyOGFP; Gal80^{ts} x DJ752UAS::CD8GFP*, *w;UASrpr.c/CyOGFP; Gal80^{ts} x w^{1118}*, *w;UASricin/CyOGFP; Gal80^{ts} x w^{1118}* . 20 larvae were pulled from each stock and placed on blue gel-agarose food overnight at 29°C. Dishes were removed from incubation the following morning, and the larvae were transferred to a plate with white gel-agarose food. The time in which it took for the blue food to be excreted was recorded after 2 hours. The experiment was repeated 5 additional times.

Larval midgut Dissection Protocol

Flies intended for study under the light microscope were dissected in a manner necessary to expose the gut of the fly completely intact with its mouthparts. Each larva was dissected and observed individually to ensure that the cells within the gut were alive and functioning as normally as possible. A larva was selected from a group with the desired genotype and placed in a well of a 9-well dissection dish. The well was filled with a sterile *Drosophila* S2 cell culture media. The larva was allowed to settle from the transfer for a period of 30 seconds. Using a pair of tweezers, the mouthparts of the larva were grabbed and held, and using a second set of tweezers the tail end of the larva was held. The mouthparts were carefully torn away from the rest of the body allowing the gut to unravel and follow. The gastric caecae attachments will most likely be torn during this

process, but overall the epithelial tube remains undisturbed. The now dissected larval gut is left undisturbed in solution for a period of 30 seconds. A slide is prepped with 500uL of drosophila media solution, and the gut is transferred carefully to the slide after the waiting period. The gut is then stretched on the slide and a cover slip is placed over the gut. The slide is then immediately transferred to the light microscope for viewing.

Morphological Measurements of the third instar larval midgut

The following genetic crosses were made from the *Drosophila* stocks discussed previously: *w;UASricin/CyOGFP::Gal80^{ts} x DJ752UAS::CD8GFP*, *w;UASrpr.c/CyOGFP:: Gal80^{ts} x DJ752UAS::CD8GFP*, *w;UASricin/CyOGFP::Gal80^{ts} x ChaGal4UAS::CD8GFP*, *w;UASrpr.c/CyOGFP:: Gal80^{ts} x ChaGal4UAS::CD8GFP*, *w;UASricin/CyOGFP::Gal80^{ts} x w^{1118}*, *w;UASrpr.c/CyOGFP::Gal80^{ts} x w^{1118}*. Flies from the *DJ752UAS::CD8GFP*, *ChaGal4UAS::CD8GFP*, and the *w^{1118}* stock lines were also measured. All fly lines were reared and prepped as stated in the ablation protocol. Following the dissection of the larval gut, measurements of the SCANS region in each *Drosophila* genotype were taken. Rather than being placed into a *Drosophila* media solution, the guts were dissected in a solution of 1% PBS, and then placed on a slide with 30% glycerol in 1xPBS. The glycerol solution provided a thicker media that was needed to mobilize the gut from moving around on the slide. Once on the stage of the light microscope, the image was focused and a photo was taken of the SCANS region. A collection of measurements from 20 larvae of each genotype were taken and recorded. The first set of measurements is the entire width of the SCANS region (Figure 4a.), the

second measurements are of the width of the lumen inside the intestinal wall (Figure 4b.). The third set of measurements consists of one side of the intestinal wall where the LHC are/used to be present (Figure 4c). After all measurements for the 20 larvae were recorded for each genotype, the wild-type data was compared to the experimental groups in a T-test assuming equal variances to determine if there was a significant difference. A difference is considered significant with a p-value of less than 0.05, and the difference is highly significant when the p-value is less than 0.001. The means and the standard deviations were calculated using the built-in formula in Microsoft Excel.

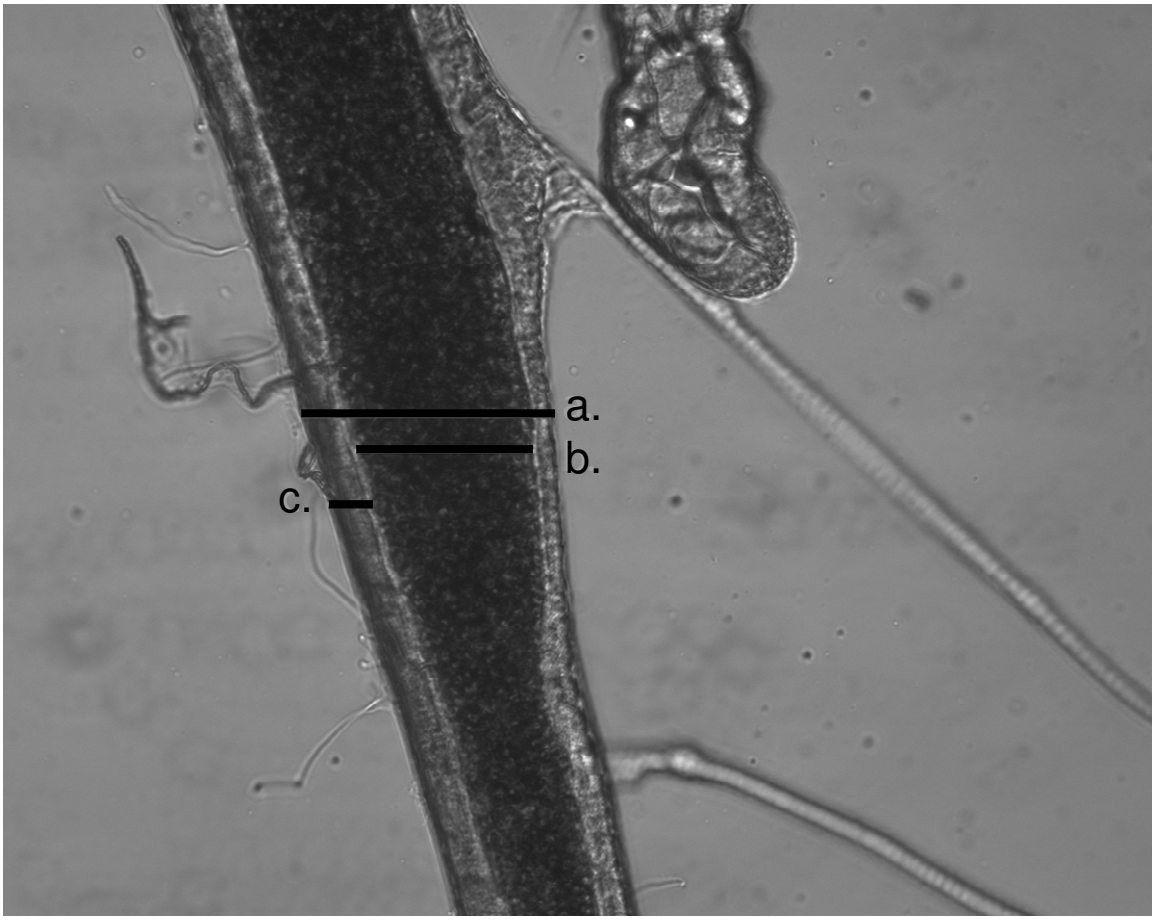


Figure 4: Measurements of the SCANS region took place below the first attachment of the gastric caecae. a. Measurement of the width of the entire gut. **b.** Measurement of the gut lumen. **c.** Measurement of gut wall.

Measuring Gut Contractions

Larval midguts from the crosses *w;UASricin/CyOGFP::Gal80^{ts} x DJ752UAS::CD8GFP*, *w;UASrpr.c/CyOGFP:: Gal80^{ts} x DJ752UAS::CD8GFP*, and *w^{1118}* stock flies were dissected after having been incubated at 29°C overnight for the experimental samples, and at room temperature for the controls. This time was shown to be optimal period for the ablation of the LHC by both ectopic expression of *ricin* and the apoptotic gene *reaper*. Midguts from one larva were dissected and analyzed individually one at a time in the following manner: dissected midgut was placed in a drop of S2 cell media on a microscope slide and a coverslip was mounted with clay feet. The SCANS region of the midgut was identified by the location of the muscular caecal tethers. A sequence of images of 100 frames was taken by the image capture program, Image Pro Plus, to time a period of 60 seconds. It was essential to ensure enough exposure time to clearly see the SCANS region of the gut during imaging for each specimen. Each twitch, rolling motion, or spasm occurring below the first muscle attachment of the gastric caeca, where the LHCs are or would be, was counted. The observation of the twitches were observed in real time through the microscope objectives, and the live preview on the computer served as a back-up and record of the data being collected. 20 larvae were observed and recorded for each genotype group examined in this experiment. As an additional control, the experiments were repeated again rearing all the fly lines on blue food at room temperature. The wild-type data was compared to the experimental group in a T-test assuming equal variances to determine if there was a significant difference. A difference is considered significant with a p-value of less than 0.05, and the difference is

highly significant when the p-value is less than 0.001. The means and the standard deviations were calculated using the built-in formula in Microsoft Excel.

Gain of Function

UASChR2II+III male flies were crossed with both *ChaGal4UAS::CD8GFP* and *DJ752UAS::CD8GFP* virgin female flies (Figure 5). The flies were reared on standard cornmeal and transferred daily. The eggs were watched carefully and upon hatching into larvae, 50uL of 100% all-trans retinal was mixed in with the cornmeal. The larvae were allowed to feed and grow on the cornmeal + retinal until day 4 after egg laying. The larvae were collected per protocol and separated under the fluorescent microscope. *UASChR2II+III x ChaGal4UAS::CD8GFP* had both brain, brainstem, and LHC expressing GFP. The *UASChR2II+III x DJ752UAS::CD8GFP* larvae have GFP expressing in the LHC and the salivary glands. About 20-30 larvae from each group were collected and placed onto small dishes containing a blue food mixed with a volume of 100% all-trans retinal (100 μM final concentration). The volume of retinal was determined by the weight in grams of the food. Food dishes were weighed and the weight of the empty food dish was subtracted from the total weight. A volume of retinal 10X the weight of the food was mixed with the food. The larvae were allowed to develop for an additional day on the more potent retinal-infused food.

The larvae from each genotype group were dissected individually per fly dissection protocol. Upon allowing the larval specimen to settle on the stage of the microscope for 30 seconds, the SCANS region was found and focused on. The "live

preview" option was selected and the exposure time adjusted. A sequence recording length of 100 frames (60 seconds) was selected. For each genotype 40 sequences, or movies, were taken. During half of the movies, the larvae were exposed to white light for the first 100 frames, and then to blue pulsating light for the next 100 frames. The other 20 movies for the genotype began with 100 frames of blue pulsating light, and ended with 100 frames of white light. Overall, 20 groups of white light sequences, and 20 groups of blue light sequences were collected for both *ChaGal4* and *DJ752*. Once data collection was completed, the number of contractions seen below the first gastric caeca muscle attachment were counted in every sequence. The data was placed into a chart, and the number of twitches seen when exposed to white light, was compared to those seen when exposed to blue light. The difference between *ChaGal4* and *DJ752* was also recorded. The data was compared in a T-test assuming equal variances to determine if there was a significant difference. A difference is considered significant with a p-value of less than 0.05, and the difference is highly significant when the p-value is less than 0.001. The means and the standard deviations were calculated using the built-in formula in Microsoft Excel.

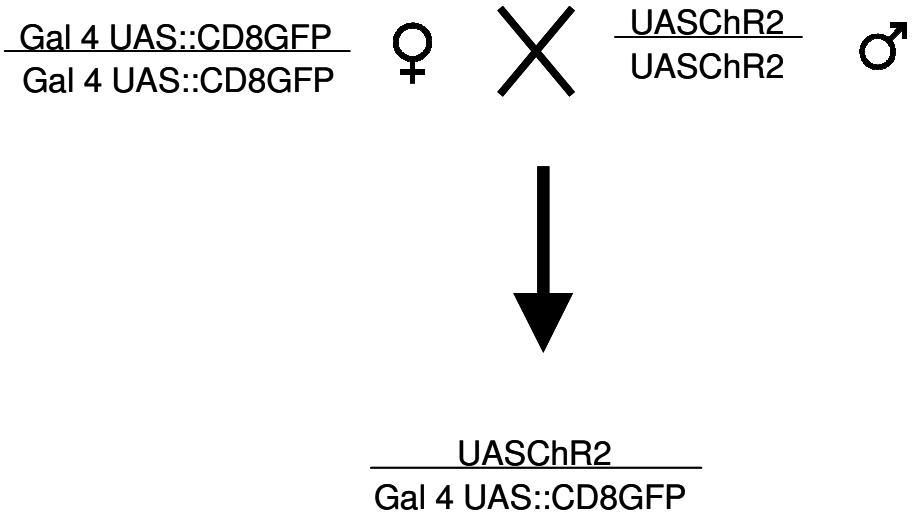


Figure 5: Map of genetic cross for gain of function experiment. One of two *Gal 4* driver lines, either *DJ752* or *Cha*, were used for female flies. The male *channelrhodopsin-2* genotype contained two copies of the *UAS::ChR2* transgene. The offspring of this cross will result in both *channelrhodopsin-2* being expressed where the *Gal 4* driver line express GFP.

CHAPTER III

RESULTS

Gal4 Enhancer Trap Screen for Midgut Expression

A variety of enhancer traps (Table 1) were acquired from the *Drosophila* stock center in Bloomington Indiana. The *Gal 4* lines were crossed with *UAS GFP* lines in order to see the expression patterns in the midgut. For each *Gal 4* line the cytological location, gene/protein (if known), and expression pattern in midgut was recorded (Table 1). Out of the 37 *Gal 4* lines screened, 21 of them express GFP in the midgut from the head of the proventriculus to the acid secreting area of the gut. (Figure1 - Midgut). Out of these 21 lines, 17 express in a cluster of oddly shaped cells at the junction of the midgut before the acid secreting portion, 5 of which, the midgut expression is exclusively in these cells alone (Table 1). These cells are long and bottle shaped and project an apical lamellipodial head into the lumen of the gut. The membranous ruffles and folds of the apical head region provided the cells their name, Lettuce Head Cells (LHC). A majority of the *Gal 4* lines that express in the LHC also have expression patterns in the proventriculus, express in the endothelial lining of the gut, and/or in stem cell crypts throughout the midgut (Table 1; Figure 6). At least five of these lines show expression in the LHC and either longitudinal muscles or circular muscles (Table 1; Figure 6a.,b.,e.,m.). With the overwhelming number of *Gal 4* lines that express in the LHC of

the midgut, I speculated that this region of the gut serves a very important purpose in the control of digestion. Ultimately, cloning one of these genetic markers would lead to a better understanding of what genes are required for the development or function of the LHC.

Table 1: Gal4 lines that express in larval midgut

Gal4 Line	**Cytological location**	**Gene/protein**	**Expression Pattern in Midgut**
Cha-Gal4	3L,91C5	*Choline acetyltransferase,* encodes an choline O-acetyltransferase and is involved in acetyl choline biosynthesis	LHC
Ddc-Gal4	2R, 37C1	*Dopa decarboxylase* encodes an aromatic amino-acid decarboxylase which plays important roles in synthesis of neurotransmitters such as serotonin and dopamine from amino acid precursors.	LHC
DJ752	3L, 96F	*Enhancer of Split/HLHm7*	LHC
Bab-Gal4	3L, 61E2-61F1	*bric a brac 1*encodes a transcription factor	LHC, LM, some CM, some En
MJ12	2L, 22B1-22B2	*CG17646*, encodes an ABC type 2 transporter	LHC
DJ761	3R, 85D16-85D17	*Passila(pa)* encodes a nuclear mRNA splicing factor	LHC, En
C805	2R, 48E6-48E7	*Developmental embryonic B/CG16972, RNA splicing factor*	LHC, expression pattern in Pro
CB20	2nd chromosome	*NA*	LHC, expression pattern in Pro
5053A	3L, 76C1	teyrha-meyrah, protein of unknown function	LM
Drm-Gal4	2l, 24C1	*Drumstick*, encodes a small C2H2 zinc finger protein	SCANS/LHC, expression pattern in Pro
DJ691	3rd chromosome	*NA*	LHC, crypt
DJ717	3rd chromosome	*NA*	Crypts/stem cells, LHC, pattern Pro, trachea, Gob
CB30	2nd chromosome	*NA*	CM, LHC, Endo, crypt
c564		*NA*	LHC, En
EDTP/DJ694	2R, 54B7-54B15	Encodes an egg-derived tyrosine phoshatase	En
MJ33a	3rd chromosome	*NA*	Expression pattern in Pro, LHC, FG
C135	3rd chromosome	*NA*	LHC, Gob
T80	2nd chromosome	*NA*	Crypt, LHC, pattern in Pro
T155	3rd chromosome	*NA*	LHC

FG- foregut; Pro – proventriculus; LHC- lettuce head cells; LM – Longitudinal muscles; CM – Circular Muscles; Crypts – Stem cell crypts; En – endothelial lining; GC – Gastric cecae; Gob -- Goblet cells

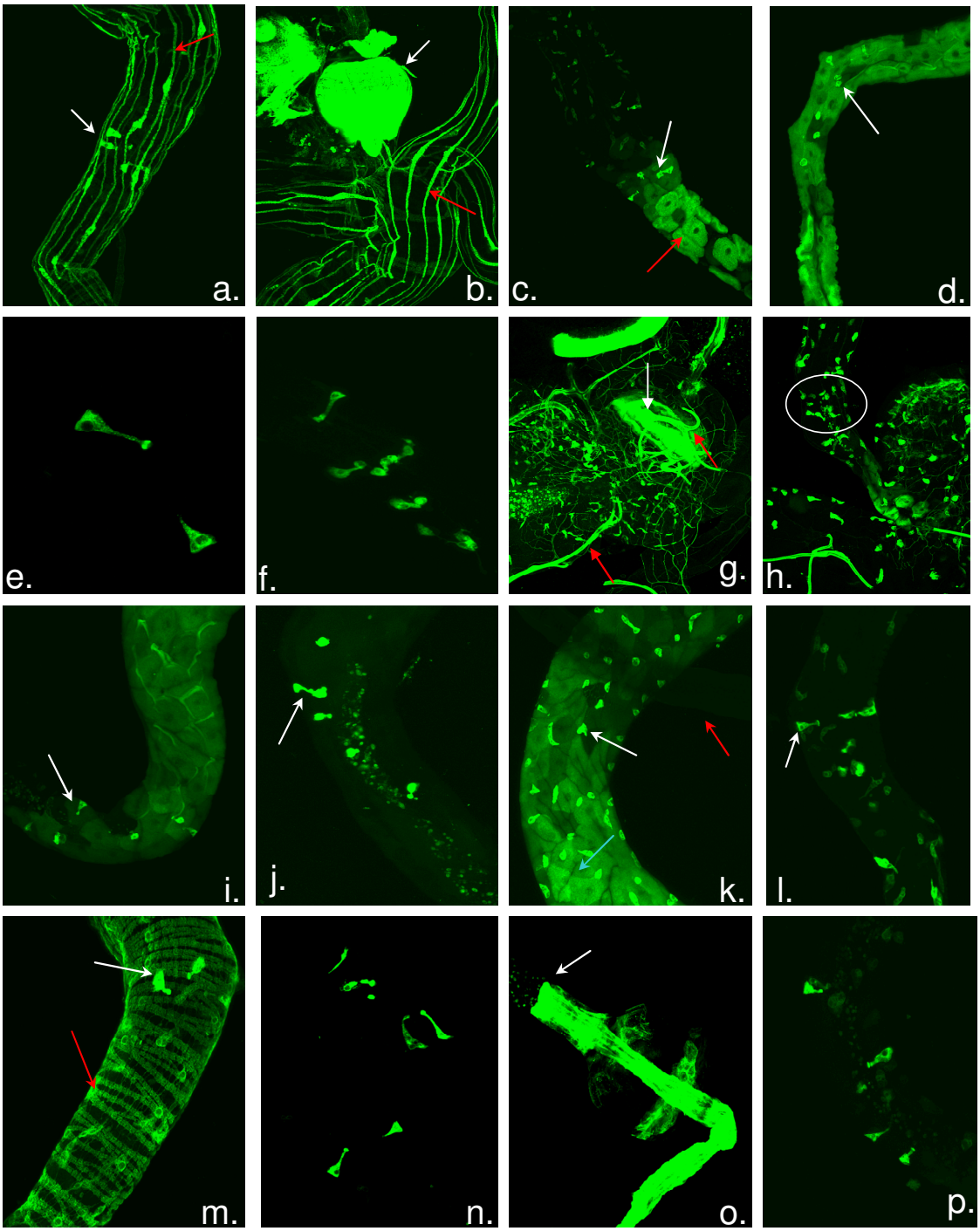

a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.
l.
m.
n.
o.
p.

Figure 6: Expression patterns of Gal 4 driver lines. **a.** *UASBabGal4* SCANS region. The white arrow indicates that this Gal 4 driver line expresses in the LHC. GFP expression in the longitudinal muscles of the midgut (red arrow). **b.** *UASBabGal4* anterior midgut GFP expression. The white arrow is indicating heavy GFP expression in the head of the Proventriculus. GFP expressing in longitudinal muscles (red arrow). **c.** *UASC135Gal4* expression in the SCANS region of the midgut. GFP expressing the the LHC group before the acid secreting portion of the gut (white arrow). GFP is also being expressed in the large copper secreting cells in the acidic portion of the midgut below the SCANS (red arrow). **d.** *UASC564GAL4* expresses in the epithelial cells throughout the midgut, but the white arrow indicates where GFP is strongly expressed in the LHC. **e.** *UASDJ752GAL4* expresses only in the LHC in 3rd Instar Larvae. **f.** *UASDdcGal4* is a driver line that labels cells that use dopamine, here in the SCANS region the LHC are clearly labeled by GFP showing us the association with the DA neurotransmitter. **g.** *UASDJ717Gal4* is heavily expressed throughout a multitude of cells in the midgut. The white arrow here is pointing to the head of the proventriculus, below this area GFP expression is seen in numerous stem cell crypts and epithelial cells. The tracheal network is also heavily labeled by GFP (red arrow). **h.** *UASDJ717Gal4* is shown at the level of the SCANS region. The portion circled in white is where the LHC are present, surrounded both above and below by various other cell types labeled by this driver line. **i.** *UASDJ761Gal4* expresses GFP throughout the midgut in the epithelial cells. The white arrow indicates the much brighter GFP expression in the LHC at the SCANS. **j.** Expression of *UASMJ12Gal4* is clearly seen in the LHC (white arrow). **k.** *UAST80Gal4* expresses in a variety of cell types in the midgut. The white arrow is pointing to heavy GFP expression in stem cell crypts in the area of the midgut above the SCANS region. The blue arrow is indicating the expression in the epithelial cells. The red arrow is pointing to a dorsal gastric caeca arm that is extending posteriorly where it will connect at the SCANS region. **l.** *UASDJ691Gal4* expression is seen in the LHC (white arrow), and various other stem cells throughout the midgut. **m.** *UASCyoCB30Gal4* expresses GFP heavily in the circular muscles throughout the midgut (red arrow). It also has heavy GFP expression in the LHC (white arrow). **n.** *UASC805Gal4* at the level of the SCANS region expresses solely in the LHC. **o.** *UASMJ33aGal4* in anterior midgut expresses GFP heavily in the gut tube that extends from the mouthparts down through the head and body of the proventriculus. The white arrow indicates where the mouthparts of the larvae are, and thus where the tube begins. **p.** *UAST155Gal4* expresses GFP heavily in the LHC as seen here at the SCANS region.

Cloning of *DJ752Gal4* Enhancer Trap

We have identified several new genetic markers for LHC in the SCANS with the *Gal 4* enhancer trap lines, known as *DJ752, C805* and *MJ12,* which express specifically in the LHC of the midgut. Of these three, *DJ752* is the most interesting and unique because it is only expressed in LHC of third-instar larvae. This finding suggests that it might be important for the function and/ or development of these cells. To clone the gene associated with the *DJ752* enhancer trap, I used the plasmid rescue technique (Brand and Perrimon, 1993) (Figure 2). Four independent clones were obtained after the colonies with the plasmid were purified and sequenced. All four clones yielded the same genomic sequence of 363 bases on chromosome 3 (Figure 7a.). One of the clones contained a chimera, in addition to the 363 base pairs, containing DNA from chromosome 4. Due to the fact that all of the clones contained a common set of bases from chromosome 3, I concluded that the DNA from chromosome 4 was a cloning artifact. This was verified by the fact that when the plasmid was cut with EcoRI, a fragment with chromosome 4 sequence distinct from the 363 base pair fragment, was generated. This band was evidence of *EcoRI* cutting the plasmid at another restriction site.

The 363 bp sequence (Figure 7a. - yellow). was used in a Blast analysis of the 363 bases (Figure 7b.- yellow). This resulted in identifying the *DJ752 Gal 4 pGawB* insertion as an element in the *Enhancer split complex* [*E(spl)*] which is approximately 2,000 base pairs upstream of the *HLHm7* gene (Figure 7c.).

Figure 7: Sequence, Alignment and Genomic site of insertion of the *DJ752 Gal4 pGawB* insertion.

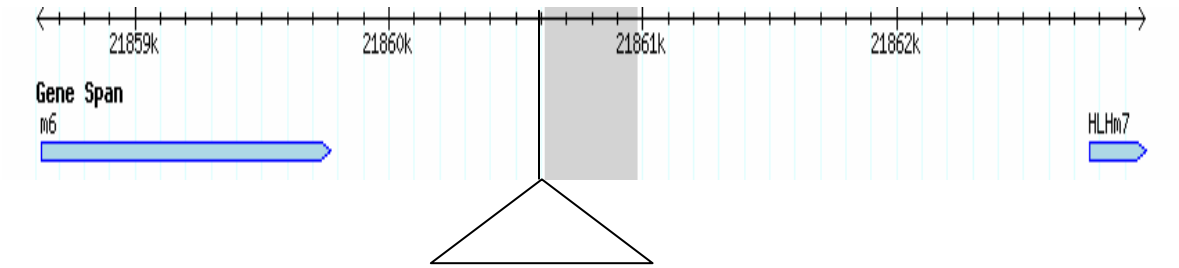
a. Sequence from plasmid rescue of *DJ752 Gal4 pGawB* (yellow - genomic/purple - P-element vector)

CATAGGTACACAATCATATCGCTGTCTCACTCAGACTCAATACGACACTCAGAATACTATTCC
TTTCACTCGCACTTATTGCAAGCATACGTTAAGTGGATGTCTCTTGCCGACGGGACCACCTTAT
GTTATTTCATCATGGCCCTGGGTTTCGTTTCGGATGGCATCGGTTGGGATCGTCCATCGCTAAG
TGGCATACATATACGCGTATGGTATTCCTGCTCACGTCGGGACATCATCATCGTGGGACCCAG
GTCCCAGAAAAAGGGATGATTGTTCGATGGATCGACGAGGGACATGCAAACTAAATGACTGT
ACGGCAAATTGACACACACTAAAGATTAAATGTATCGCATTCGTTAACTATATAAACAATATA
CGTGGCCGGTTTGAGGAACGCTGGCTGCGGCAGGAAGTGTGTGTGTGCAGTGCACTGCGTGCT
GAGAGTGTGCTGAACAACGGGCCGTTACAATTTTTCTGGCGACTGTGAGAAAATTTCTCGAGG
GGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCG
TCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCAC
ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGT
TGCGCAGCCTGAATGGCGAATGGAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAA
TTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCA
AAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAA
GAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTC

b. Alignment of the 363 bp of 3' genomic DNA flanking the *DJ752 Gal4 pGawB* insertion with genomic DNA from the *Drosophila melanogaster* 3rd chromosome

```
DJ752 3' flank:141        GGCCCTGGGTTTCGTTTCGGATGGCATCGGTTGGGATCGTCCATCGCTAAGTGGCATACA  200
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome:21860977   GGCCCTGGGTTTCGTTTCGGATGGCATCGGTTGGGATCGTCCATCGCTAAGTGGCATACA
21860918
DJ752 3' flank:201        TATACGCGTATGGTATTCCTGCTCACGTCGGGACATCATCATCGTGGGACCCAGGTCCCA  260
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome: 21860917  TATACGCGTATGGTATTCCTGCTCACGTCGGGACATCATCATCGTGGGACCCAGGTCCCA
21860858
DJ752 3' flank:261        GAAAAAGGGATGATTGTTCGATGGATCGACGAGGGACATGCAAACTAAATGACTGTACGG  320
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome: 21860857  GAAAAAGGGATGATTGTTCGATGGATCGACGAGGGACATGCAAACTAAATGACTGTACGG
21860798
DJ752 3' flank:321        CAAATTGACACACACTAAAGATTAAATGTATCGCATTCGTTAACTATATAAACAATATAC  380
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome: 21860797  CAAATTGACACACACTAAAGATTAAATGTATCGCATTCGTTAACTATATAAACAATATAC
21860738
DJ752 3' flank:381        GTGGCCGGTTTGAGGAACGCTGGCTGCGGCAGGAAGTGTGTGTGTGCAGTGCACTGCGTG  440
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome: 21860737  GTGGCCGGTTTGAGGAACGCTGGCTGCGGCAGGAAGTGTGTGTGTGCAGTGCACTGCGTG
21860678
DJ752 3' flank:441        CTGAGAGTGTGCTGAACAACGGGCCGTTACAATTTTTCTGGCGACTGTGAGAAAATTTCT  500
                          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3rd chromosome: 21860677  CTGAGAGTGTGCTGAACAACGGGCCGTTACAATTTTTCTGGCGACTGTGAGAAAATTTCT
21860618
DJ752 3' flank:501        CGAG  504
                          ||||
3rd chromosome: 21860617  CGAG 21860614
```

c. Genomic view of the *DJ752 Gal4 pGawB* insertion on the 3rd Chromosome in the *E(spl)* complex



DJ752 pGawB P-element insert 363bp sequence of flanking DNA

Defining the SCANS Region of the Larval Midgut

We have identified a novel component of the *Drosophila* autonomic nervous system within the larval midgut, which we call the Superior Cuprophillic Autonomic Nervous System (SCANS). The SCANS region is located at the juncture between the copper cell/acid secreting region and the distal portion of the anterior midgut (Figure 8a.) and is characterized by three structures: (1) a muscular valve region devoid of large cuboidal endothelial cells (Figure 8a.); (2) a cluster of 7-9 neuron-like Lettuce Head Cells (LHC) (Figure 8a.); and (3) a muscular connection to the anterior midgut via specialized longitudinal muscles that project from the tips of the dorsal gastric caeca (Figure 8b.- e.). The organization and structure of the SCANS suggests that it may function to govern and coordinate the functions of the larval anterior midgut as a whole.

Another novel feature of the SCANS is the association of the tips of the dorsal gastric caeca with the region itself. This discovery conflicts with the currently accepted organization of the gut, which has all four gastric caeca extending anteriorly towards the mouthparts inside the larva (Demerec, 1965). The gastric caeca are four diverticula, two ventral and two dorsal, which project from the very anterior midgut just posterior to the proventriculus. We have found that the two dorsal caeca initially extend forward but soon double back and form a connection to longitudinal muscles at the SCANS region (Figure 8c. and e.). This longitudinal muscle, where the connection occurs, is a specialized muscle (Figure 8b.) that expresses high levels of the junctional protein Disc Large and distinguishes the dorsal from ventral gastric caeca (Figure 8c. and d.). This specialized muscle cell structure has non-ordered sarcomeric organization (data not

shown) and an unusual localization of DLG in large intracellular plaques that run the length of the muscle cell.

Our results suggest that the dorsal gastric caeca may communicate with the lower anterior midgut through the SCANS region to regulate the flow of food through the gut. The organization and structure of the SCANS suggests that it may function to govern and coordinate the functions of the larval anterior midgut as a whole.

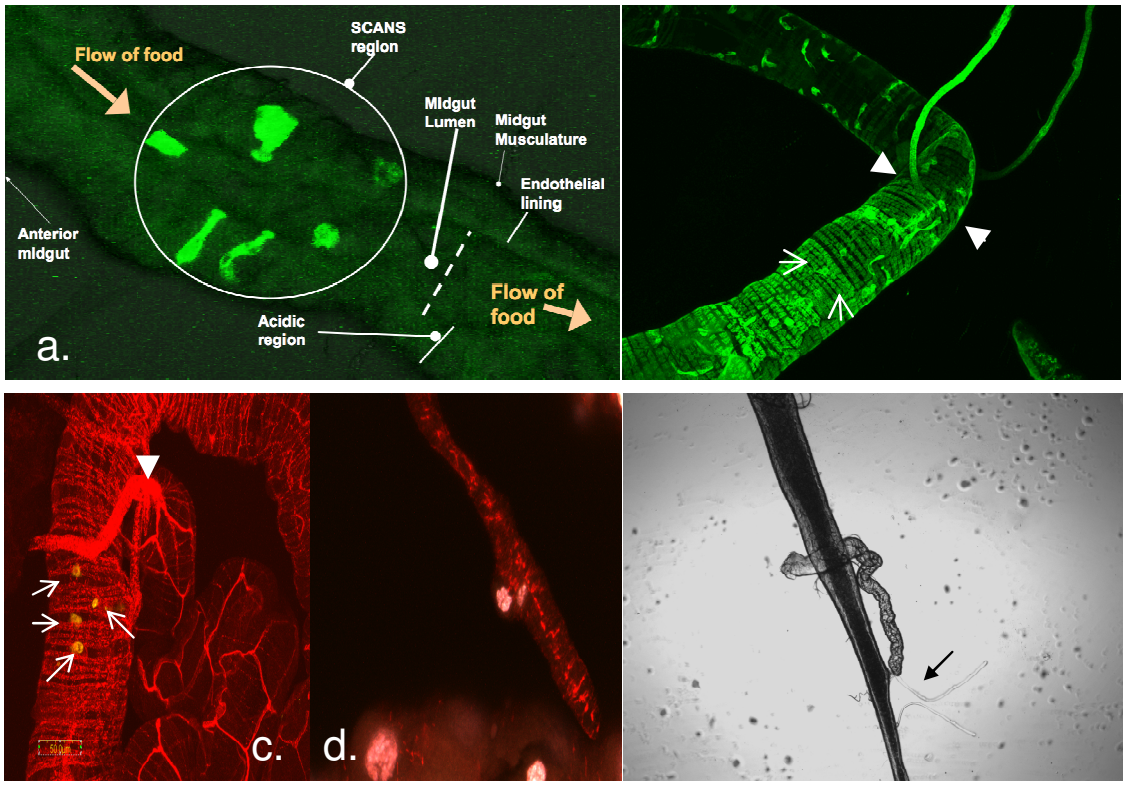


Figure 8 – SCANS. **a.** The SCANS region is an area above the acidic region of the midgut and an area below the anterior-most portion of the midgut that contains the proventriculus. The circled portion represents the actual SCANS region that contains the electrically active LHCs. In this picture the lumen and the gut walls containing musculature that extend the length of the intestines are clearly visible. **b.** The SCANS portion of the midgut shown here is found between the two longitudinal muscles that extend out from the gut wall that connect to the gastric caeca (arrow heads)**.** Circular muscles that extend the length of the midgut through the SCANS region are also visible (arrows). **c.** *Drosophila* larva fixed and labeled with anti-Disc Large (DLG) antibody in red. Arrows point to the lettuce head cells of the SCANS region. A newly discovered connection between the gastric caeca and the SCANS region of the midgut can be observed. A specialized muscle cell structure emanates from the tip of the gastric cecae into the SCANS region (large arrow head). **d.** This specialized muscle cell structure has non-ordered sarcomeric organization (data not shown) and an unusual localization of DLG in large intracellular plaques that run the length of the muscle cell. **e.** A light microscope picture of a larval midgut. The SCANS region is below the attachment of the first dorsal gastric caeca that actually wraps around the gut wall and connects to the longitudinal muscle indicated by the arrow. The lumen of the midgut is dark because it is filled with blue food.

Structural Characterization of the LHC

The most interesting feature of the SCANS region in the larval midgut is the lettuce head cells (LHC). These long, bottle shaped cells appear to be neuronal in nature as they express a number of different nervous system markers including *Choline actyltransferase* (*Cha*) and *Dopa decarboxylase* (*Ddc*) (Figure 6f.and Table 1). Each LHC extends through the endothelial epithelium and appears to be associated either directly or indirectly with an overlying longitudinal muscle (Figure 9d.). LHC project an apical lamellipodial head that has membranous ruffles and folds into the lumen of the gut (Figure 9b. and c.). Their position within the midgut wall is much like well known enteroendocrine cells which have their basal membrane attached to the visceral muscles, and an apical projection into the luminal portion of the gut surrounded by the absorptive villi of the epithelial lining (Figure 9a.).

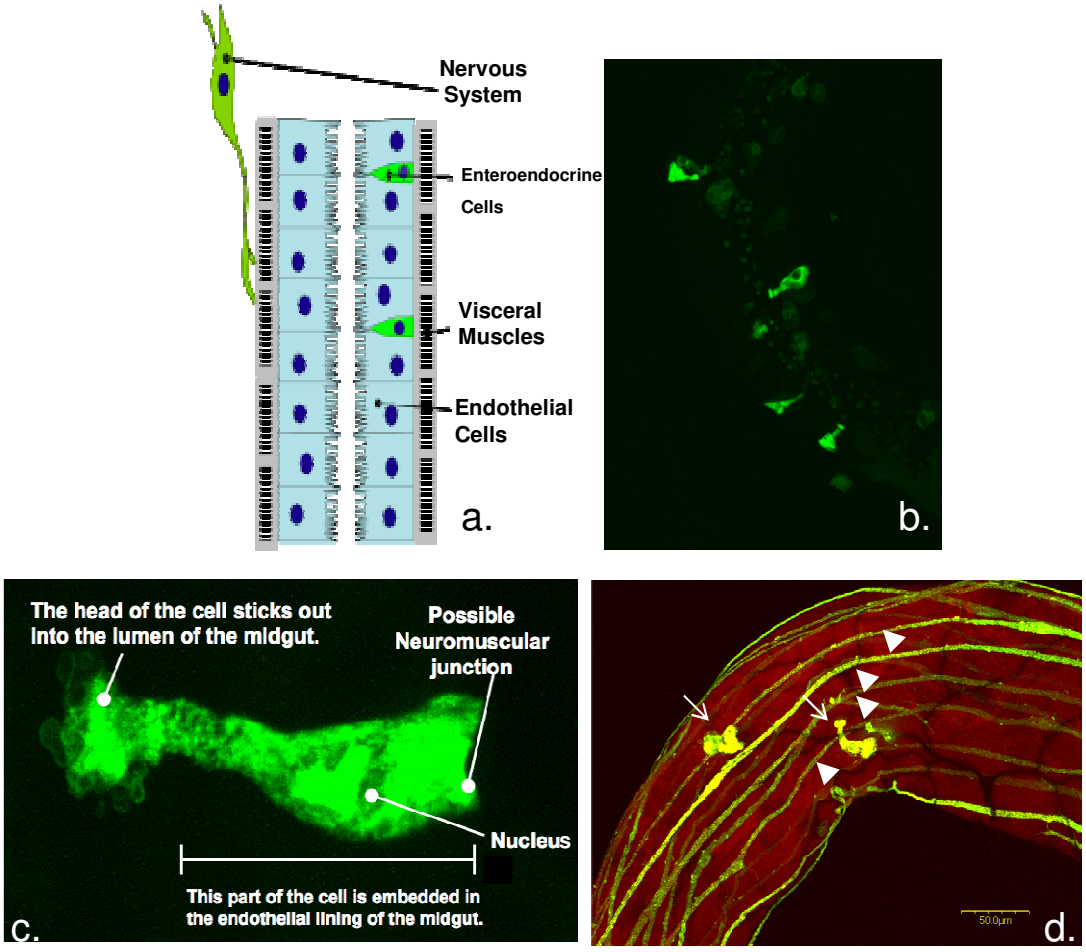


Figure 9: LHC Cells and Organization. **a.** Schematic of the *Drosophila* midgut. **b.** 7 of the LHC and their orientations in the gut wall. **c.** A live LHC at a 60X magnification using an oil immersion objective. Here you can clearly see the ruffled luminal lamellipodial head, the nucleus, and the basal end of the cell that might have neuromuscular junction with visceral gut muscle. **d.** LHC (arrow) lie directly beneath the longitudinal visceral muscles (arrow heads).

Functional Characterization of the Requirements of LHC in SCANS Function

The discovery of the SCANS region of the *Drosophila* midgut, along with the physical and genetic characterization of the LHC lead me to hypothesize that they are required for peristaltic movement in the digestive tract. This is based on the fact that the LHC seem to have a connection to a variety of muscle types, and secrete neurotransmitters that are involved in muscular contraction (Ach) and appetite control (DA). To address the potential role of the LHC in the SCANS region functionality, the following experiments were conducted.

LHC Ablation Experiments

The first set of experiments focused on removing the LHC from the SCANS region and then evaluating the morphology and function of this region. The first approach is a cell ablation technique that involves expression of a cytotoxin in the LHC (Hidlago et al., 1995; Duffy 2002). The cytotoxins used for the experiment were expressed with the *Gal4/Gal80^{ts}* system, which allows not only spatial restriction (i.e., in the LHC), but also temporal control of the toxin (Zeidler et al., 2002; Suster et al., 2004). The function of the larval midgut without the LHC was assessed in three separate assays: (1) a feeding assay in which the speed that food is moved through the gut was measured; (2) a morphological assay in which we measured the width of the lumen, the thickness of the side-walls, and overall width of the gut at the SCANS region in the midgut; (3) a functional assay in which we observed the peristalsis and muscle contraction in this region of the gut. A group of 20 larvae for each genotype was assayed for each experiment. The LHC ablated

larvae were generated by crossing female flies carrying *UAS::Cytotoxin* and *Gal80^{ts}* with male flies carrying a *Gal4 UAS::CD8GFP* enhancer trap line (Figure 3). The progeny of this cross was raised at room temperature and then larvae were collected on the fifth day after egg-laying. We have determined this day to be the optimal for two reasons: the larvae are large enough to manipulate and dissect easily and the larvae are still actively feeding. The larvae were placed on food dyed with bromophenol blue, that when ingested allows for the alimentary canal to be easily observed upon dissection. Then, the larvae were placed in an incubator overnight at 29°C. Rearing the larva at this temperature results in the loss of *Gal80 ^{ts}* suppression of *Gal4* mediated transcription and thus the *UAS::Cytotoxin* (along with *UAS::CD8GFP*) is expressed in the LHC via the *Gal 4* driver line. The controls have shown that just the *UAS::CD8GFP* is expressed under these conditions. Furthermore, the immediate appearance and subsequent disappearance of the GFP marker will allow us to determine that in fact the system works: the cells express GFP and the cytotoxin, and then disappear because they die. We observe holes in the gut wall after treatment that fill with blue food indicating the location of the ablated LHC (Figure 10). The goal of these three experiments is to determine the overall functional role of the LHC in the organization and function of the midgut in the SCANS region.

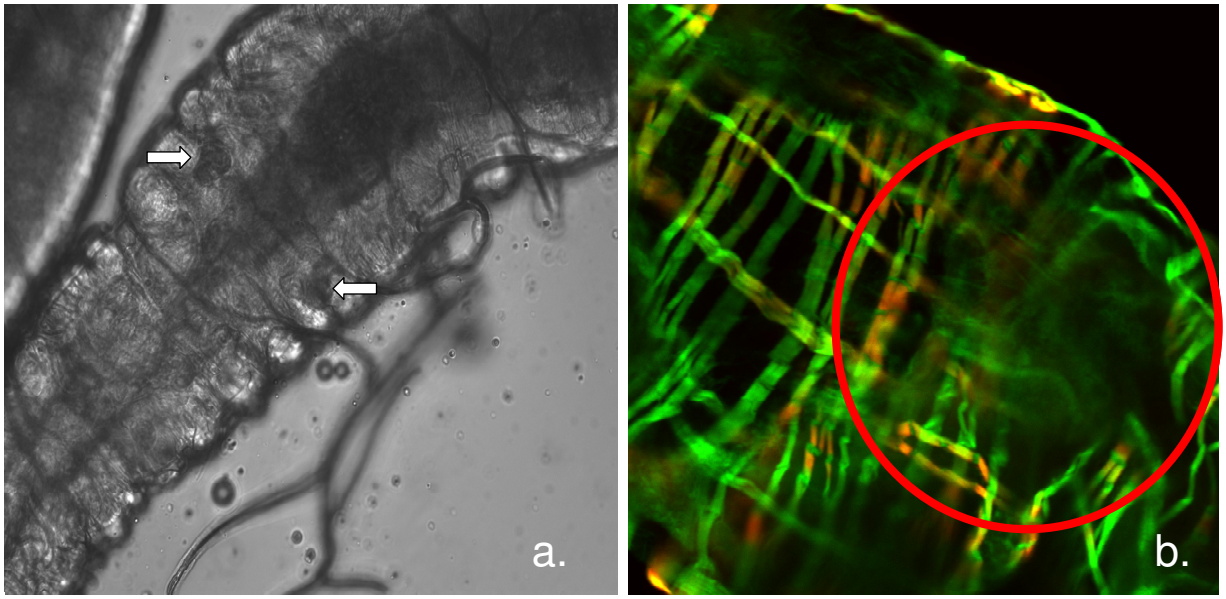


Figure 10: Suppression of *Gal80^{ts}* results in ablation of LHC via simultaneous *Gal 4* driver line *UASGFP* and *UAScytotoxin* expression. a. Arrows indicate holes in the gut wall where LHC have died. **b.** Labeling of actin (green) and myosin (red) in the SCANS region of the larval midgut. The red circle also indicates a hole in the gut wall where LHC have died.

LHC ablation does not alter movement of food through the gut

To generate larvae for the feeding assay with their LHC ablated, female flies carrying *UAS::RICIN A* or *UAS::Rpr.C* and *Gal80^{ts}* were crossed with male flies carrying either *Cha* or *DJ752Gal 4 UAS::CD8-GFP* (Figure 3). The larvae were collected and separated using a fluorescent microscope. Larvae for the experimental groups that were "non-glowing" were selected for the feeding experiment. The lack of GFP expression shows that the *Gal80^{ts}* is suppressing both the cytotoxin and the GFP, and the desired genotype is being acquired (Figure 3). 20 to 30 larvae of each genotype were placed on blue food and incubated overnight at 29°C. 8 - 12 hours later, the larvae were removed and allowed to sit at room temperature for 2 to 3 hours. During the overnight incubation at 29°C the cells targeted by the expression of the cytotoxin die. 20 larvae were transferred to white food, and allowed to eat for a period of 2 hours. After this eating period expired, each group was removed from the food and the number of larvae with white throughout their intestines was recorded along with the total number of larvae alive in the dish. For each genotype, *w^{1118}* (control), *UAS ricin/+; DJ752Gal4/Gal80^{ts}, UAS rpr.c/+; DJ752Gal4/Gal80^{ts}, UAS ricin/+; ChaGal4/ Gal80^{ts}, and UAS rpr.c/+; ChaGal4/ Gal80^{ts}* a percentage of (white larvae/total number of larvae) was calculated for each of the six feeding assays performed, and the average of those was acquired (Table 2). Only one of the experimental groups , *UAS ricin/+; ChaGal4/ Gal80^{ts},* showed a significant difference ($p<0.05 \sim 0.03$) in percentage when compared to wild-type (*w^{1118}*) in a T-test assuming equal variances (Table 2). All other experimental group's total white percentages were not significantly different when compared to wild-

type larvae, suggesting that the speed at which food moves through the gut is not affected by the disappearance of the LHC (Table 2).

Table 2: Ablation of LHC does not change speed that food moves through gut.

Genotype	n	% total white
w^{1118}	94	39%
UAS ricin/+; DJ752Gal4/Gal80^{ts}	81	44%
UAS rpr.c/+; DJ752Gal4/Gal80^{ts}	83	42%
UAS ricin/+; ChaGal4/ Gal80^{ts}	97	25% *
UAS rpr.c/+; ChaGal4/ Gal80^{ts}	96	42%

* significantly different from W^{1118} , P<0.05

Ablation of LHC in the anterior midgut does not alter the morphology of the SCANS region

Knowing that the rate of movement is most likely not affected by the ablation of the LHC, I then decided to determine whether the integrity of the gut at the SCANS region is compromised when the LHC are ablated. For this experiment a variety of crosses were set up that allowed for external controls, internal controls, and experimental groups to be compared. For the external, or wild-type controls, w^{1118}, *DJ752Gal4UAS::CD8GFP*, and *ChaGal4UAS::CD8GFP* fly stocks were used. *UAS ricin/+;* w^{1118} and *UAS rpr.c/+;* w^{1118} crosses were set up the same way as experimental groups (Figure 3), except w^{1118} was used in place of a *Gal 4* enhancer trap line. The 4 experimental groups used were prepared (Table 3) the same way as in the feeding

experiment. With the exception of the external control groups, all larvae were placed in an incubator overnight at 29°C, and the cells were allowed to die over the same time period as before. 20 larvae from each group were dissected according to protocol, and examined under a light microscope. Using image software, a picture of the SCANS region was taken for each specimen and measurements were taken as described in the materials and methods and shown in Figure 4. Since the larval gut was being maintained PBS for this experiment, sometimes the valve portion of the SCANS was caught closed. Open lumens were recorded as "1"'s, and closed lumens recorded as zeros, and the percentage of total open lumens, representing an open valve, for each genotype was calculated (Table 3). The averages and standard deviations for every measurement of each genotype group were calculated and compared (Table 3) to the controls in a T-test, to see if the morphology of the SCANS region of the larval midgut had changed due to the loss of the LHC. Every experimental group, when compared to w^{1118}, showed no significant difference in outer diameter, inner diameter, or the ratio between the two (Table 3). The width of the intestinal wall was significantly ($p< 0.05$) smaller in three out of four of the ablated fly lines (Table 3, *) when compared to w^{1118}, *DJ752Gal4*, and *ChaGal4* external control flies. These results do not remain the same when the experimental groups are compared to the internal control groups, and in this instance no significant difference between any of the measurements is seen across the board (Table 3). The open lumen percentage difference again was significant when compared to the external, wild-type, control groups (Table 3, *) in two of the experimental situations, but when compared to the internal wild-type x cytotoxin crosses, there was no difference.

Table 3: Ablation of LHC in the anterior midgut does not alter the morphology of the SCANS region.

Genotype	**Condition**	**n**	**Outer diameter of SCANS ±SD**	**Inner diameter of lumen ±SD**	**Ratio: Outer/Inner diameter ±SD**	**Width of intestinal wall ±SD**	**% of Open Lumen ±SD**
w^{1118}	External Control	20	339.2±52.8	123.3±69.3	0.38±0.21	128.2±51.5	60%
DJ752Gal4UAS::CD8GFP	Control	20	341.2±107	129.1±60.7	0.39±0.17	121.9±54.9	65%
CHAGal4UAS::CD8GFP	Control	20	268.1±53	51.3±64.1	0.18±0.21	120.1±39.6	45%
UAS ricin/+; w^{1118}	30°C o/n; RT 3 hours	20	329.1±69.3	204.7±102.5	0.59±0.24	67.5±26.1	80%
UAS rpr.c/+; w^{1118}	30°C o/n; RT 3 hours	20	311.3±71.3	169.8±89.9	0.52±0.21	87.9±34.2	85%
UAS ricin/+; ChaGal4/ $Gal80^{ts}$	30°C o/n; RT 3 hours	20	326.1±116.8	152.4±106	0.42±0.17	95.7±27.4*	80%
UAS rpr.c/+; ChaGal4/ $Gal80^{ts}$	30°C o/n; RT 3 hours	20	321.1±85.3	148.9±86.9	0.45±0.20	103.1±39.5 *	90%*
UAS ricin/+; DJ752Gal4/$Gal80^{ts}$	30°C o/n; RT 3 hours	20	372.3±96	155.3±64.7	0.43±0.16	137.6±63.8	90%*
UAS rpr.c/+; DJ752Gal4/$Gal80^{ts}$	30°C o/n; RT 3 hours	20	307±83.5	124.7±84.8	0.38±0.19	96.8±30.9*	80%

* significantly different from w^{1118}, P<0.05
** significantly different from w^{1118}, P<0.001

LHC ablation results in decreases in gut contractions

Gut contractions were quantified over a 60 second period under a light microscope. Following the dissection of the larval gut, the intestine was placed on a slide in *Drosophila* media, and the SCANS region observed closely for twitching. Each time the SCANS region moved it was counted. Representative photos of dissected and ablated guts imaged every 2.5 seconds are shown in Figure 11. w^{1118}, was used as the control for these experiments and *UAS ricin/+; DJ752Gal4/Gal80*^{ts} and *UAS reaper/+; DJ752Gal4/Gal80*^{ts} were used as the experimental groups. The three groups were placed under two separate conditions: 1) maintained on blue food and incubated overnight at 29°C, and 2) maintained at room temperature overnight (Table 4, conditions). At least 20 larvae were observed and contractions recorded for each group. For the groups that remained at room temperature overnight, I did not see a difference in the number of contractions per minute for either of the experimental groups when compared to w^{1118} (Table 4), which was expected. At room temperature, *Gal80*^{ts} continued to suppress both GFP and cytotoxic expression, and no LHC died. The experimental groups should be the similar, if not identical, to the wild-type control. However, when the experimental groups are incubated overnight at 29°C, there is a significant ($p< 0.001$) decrease in the number of contractions per minute when compared to w^{1118} larvae that were also incubated overnight 29°C (Table 4). The majority of the larvae that had dead LHC showed little or no contractions. When there was movement observed, it was usually in the forms of: irregular movements, rolling of the gut, and uncoordinated contractions/

random twitches. These results indicate that the LHC are required for perpetuating peristalsis between the anterior midgut and the acid secreting portion of the gut.

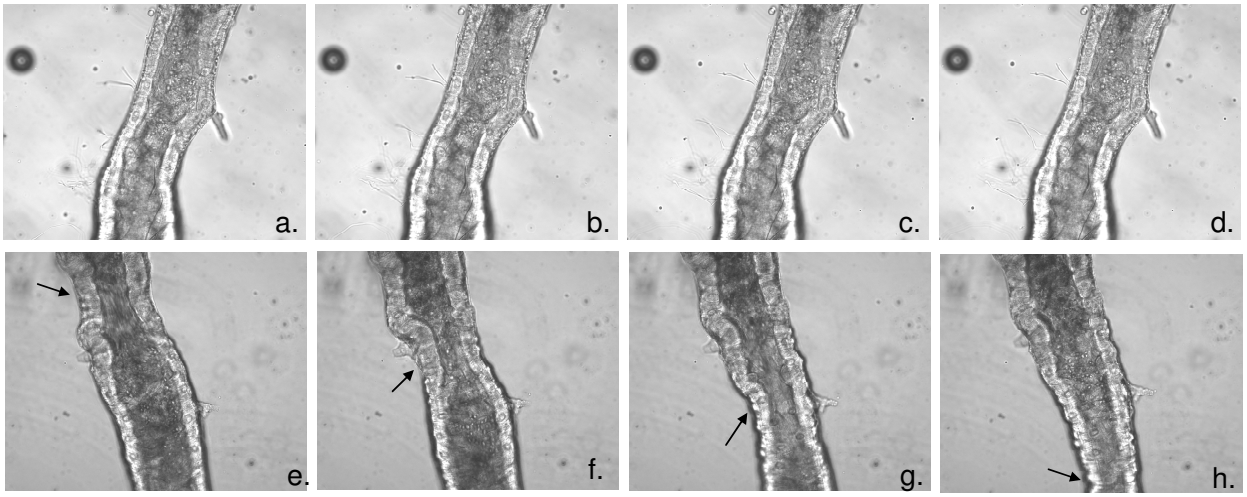


Figure 11: Contractions of the SCANS region. a-d. *UAS rpr.c/+; DJ752Gal4/Gal80ts* experimental group SCANS region over a 4 frame time lapse (approx 2.5 seconds). There are no visible contractions occuring in this area. **e-h.** W^{1118} SCANS region over a 4 frame time lapse (approx 2.5 seconds). The arrows indicate where a contraction is traveling down the length of the midgut in these pictures.

Table 4: Removal of the LHC from anterior midgut results in a loss of contractions through the SCANS region.

Genotype	Condition/Temperature treatment	n	Average # of contractions per minute ± SD
w^{1118}	29°C o/n; RT 3 hours	33	10.7 ± 3.1
UAS ricin/+; DJ752Gal4/Gal80^{ts}	29°C o/n; RT 3 hours	30	2.6 ± 2.8**
UAS reaper/+; DJ752Gal4/Gal80^{ts}	29°C o/n; RT 3 hours	20	2.0± 2.7**
w^{1118}	RT o/n; RT 3 hours	20	9.7 ± 5.4
UAS ricin/+; DJ752Gal4/Gal80^{ts}	RT o/n; RT 3 hours	20	14.35 ± 5.9
UAS reaper/+; DJ752Gal4/Gal80^{ts}	RT o/n; RT 3 hours	20	8.45 4 ± 4.5

** highly significant P<0.001

Ectopic Activation of LHC Using UASChR2 Expression

The second set of experiments involves the use of a gain-of-function assay. These experiments were carried out to see whether the LHC are sufficient to stimulate muscle contractions in the SCANS region of the midgut. To do this I expressed the *Chlamydomonas reinhardtii* Channelrhodopsin-2 (ChR2) protein in the LHC using the *Gal 4/UAS* system (Brand and Perrimon, 1993; Scholl et al., 2006). The ChR2 protein is a light-activated cation-selective ion channel that was originally found in green algae, and when expressed in electrically sensitive cell (i.e. muscle or neurons), it will initiate an action potential when exposed to a blue light λ~ 480nm (Scholl et al., 2006). By crossing the *ChR2* flies with *Gal 4* driver lines that are expressed in the LHC (Figure 5), like *ChaGal 4* or *DJ 752 Gal4*, the expression of *ChR2* was targeted to the LHC and used to

stimulate them directly with exposure to blue light. The larvae were be supplemented with 100% all-trans retinal in their food until they reach third instar as described (Scholl et al., 2006). Retinal is a necessary chemical compound needed to bind to the *Channelrhodopsin-2* ion channel in order for the channel to open and stimulate an electrical response inside of the electrically active cells in which it has been inserted (Scholl et al., 2006).

Midguts from larvae expressing the *ChR2* gene in the LHC were dissected in *Drosophila* S2 cell media and video recordings of their muscular contractions were collected, much like the w^{1118} example from Figure 11e.-h., using a camera attached to the light microscope. A series of exposures were taken over a defined time period. In the first trial, the larvae were exposed to white light for 100 frames (approximately 60 seconds) and then exposed to blue light for another 100 frames of images. The reverse experiment (blue light first and then white light) was also done. The number of contractions seen in each 100 frames was recorded along with whether the larva was exposed to blue or white light first. A total group of 40 larvae was measured for each genotype, 20 exposed to white light first, 20 exposed to blue light first (Table 5).

Individuals in the control (wild-type) group for this experiment were w^{1118}, which were raised and treated the exact same way as those in the experimental groups. While *UASChR2; Cha Gal4*, served as one of the experimental groups, it also served as an internal control, because all electrically active cholinergic cells expressing GFP should have been stimulated by the blue light. *UASChR2; Cha Gal4*, whole mount larvae were tested prior to dissection to ensure that the *UASChR2; Gal 4* system was indeed working.

The evidence of this is clearly visible in Figure 12, where all feeding motion of the larva ceases because stimulation from blue light causes the mouthparts of the larvae to retract into the head and all cholinergic neurons to contract throughout the body.

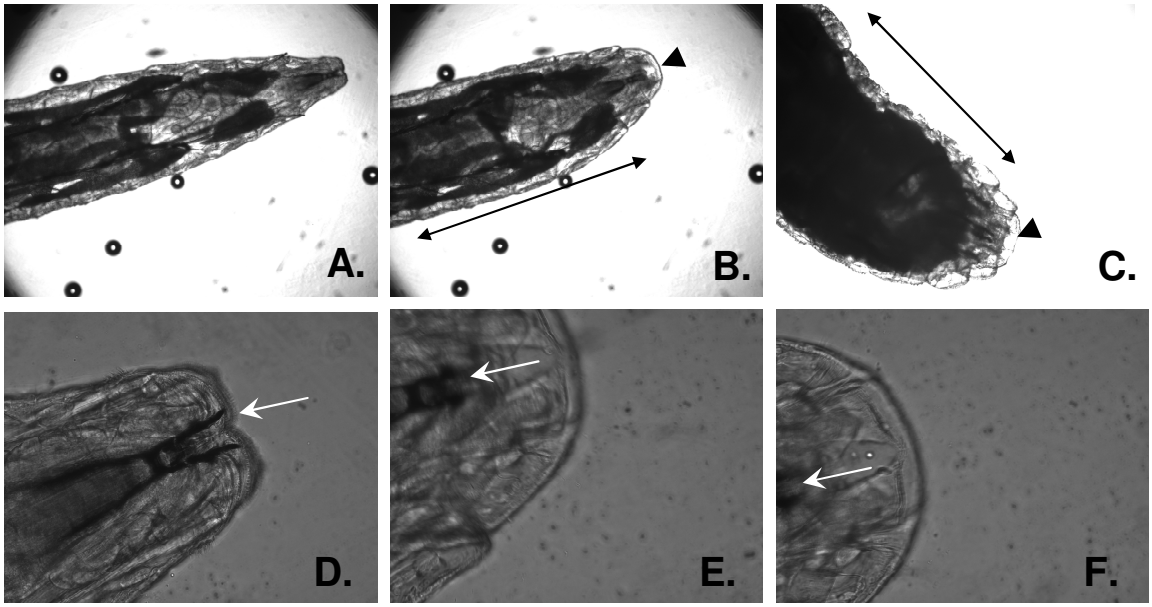


Figure 12: *UASChR2; Cha Gal4* whole mount larval contraction. **A**. Larva before blue light is turned on in normal extended feeding behavior. **B.** Larva just after stimulation with blue light. Contractions of cholinergic neurons throughout length of body (double sided arrow) and retraction of mouthparts are seen (arrow head). **C.** Larva after a few seconds under blue light stimulation. Stronger contractions of cholinergic neurons throughout length of body (double sided arrow), retraction of mouthparts (arrow head), and rotation of the larva are all seen with perpetual stimulation. At this point feeding behavior has ceased because of contraction. **D.** 400X magnification of larval mouthparts (arrow) under white light. **E.** 400x magnification of larval mouthparts just after stimulation with blue light. Mouthparts are retracted during stimulation (arrow) **F.** 400x magnification of larval mouthparts after a few seconds under blue light stimulation. Mouthparts are retracted further with perpetual blue light stimulation (arrow). At this point feeding behavior has ceased because of contraction.

The *UASChR2; DJ752 Gal4* experimental group, when exposed to blue light first, showed a highly significant ($p< 0.001$) increase in the average number of contractions per minute when compared with *UASChR2; DJ752 Gal4* exposed to white light first (Table 5, **). *UASChR2; Cha Gal4* larvae, when exposed to blue light first, had significantly ($p< 0.05$) more contractions per minute than *UASChR2; Cha Gal4* larvae exposed to white light first (Table 5, *). However, the number of contractions in the *UASChR2; Cha Gal4* larvae exposed to blue light first were did not differ from the w^{1118} controls. On the other hand, *UASChR2; DJ752 Gal4* larvae exposed to blue light first not only displayed a significant increase when compared to the same genotype exposed to white light, but the same level of significance remained when it was compared to the w^{1118} controls (Table 5).

The exposure to blue light results in a greater frequency of contractions than the white light. From these results and the ablation experiments I am able to conclude that the LHC is responsible for the initiation and/or propagation of muscular movements through the SCANS region of the larval midgut. Whether these cells have a direct interaction with the muscle, or indirect, remains unclear.

Table 5: Activation of the LHC is sufficient to induce ectopic contractions in the SCANS region.

Genotype	**Condition**	**n**	**Average # of contractions per minute ±SD**
w^{1118}	White Light	20	12.7 ±5.5
w^{1118}	Blue Light	20	12.8 ±2.7
UASChR2; DJ752 Gal4	White Light	20	11.5 ±4.2
UASChR2; DJ752 Gal4	Blue Light	20	16.4 ±4.0 **
UASChR2; Cha Gal4	White Light	20	9.7 ±4.5
UASChR2; Cha Gal4	Blue Light	20	12.2 ± 4.0 *

* significantly different from control white light, $P<0.05$

** significantly different from control white light, $P<0.001$

CHAPTER IV

DISCUSSION

Overview

The *Drosophila* larval midgut consists of a two thin layers of visceral muscle that sheath an endothelial tube. The endothelial tube itself consists primarily of large absorptive endothelial cells that arise from stem cell crypts. Midgut stem cells generate two classes of general cell types: absorptive endothelial cells and enteroendocrine cells. Although the proventricular/ anterior portions of the larval midgut have direct input from the CNS, the distal midgut appears to be disconnected from the CNS. The mechanism that maintains and propagates peristaltic muscle action throughout the rest of the gut is unknown. In a screen of 37 *Gal4* enhancer traps lines, I have identified 21 lines that express in a cell specific pattern within the larval midgut. The goal of these experiments was to identify and characterize functional regions within the larval midgut to better understand the mechanisms that govern the movement of food through the alimentary canal. As a result of this screen, we have also identified a novel region that we call the Superior Cupric Autonomic Nervous System or SCANS and have shown that the LHC are specific to this region and are required for muscle contraction in this region of the midgut.

The SCANS Region Defined

The SCANS region is located at the juncture between the distal portion of the anterior midgut and the copper cell/acid secreting region. The SCANS is characterized by three structures: (1) a muscular valve, (2) a cluster of 7-9 LHCs and (3) a connection to the anterior midgut via specialized longitudinal muscles that project from the tips of the dorsal gastric caeca. LHCs express a number of different nervous system markers including *Cholineactyltransferase* and *Dopa decarboxylase*.

Each LHC extends from a contact with a longitudinal muscle through the endothelial wall of the intestine below and project a lamellipodial head into the lumen of the gut. The LHC organization, localization and function suggest that they may be *Drosophila* analogues to the Interstitial Cells of Cajal (ICC) from vertebrate autonomic nervous systems and coordinate muscle contractions and other functions of the entire anterior midgut. The organization and structure of the SCANS suggest that it may govern and coordinate the functions of larval anterior midgut as a whole. By identifying the gene product of the *DJ752* enhancer trap line, we better understand that these genes are required for the development and function of the LHC. This cloning also assists in understanding the genetic features that distinguish the LHC from the rest of the larval midgut. The cell body of the LHC is long and skinny and extends through the endothelial lining of the midgut. The distal end of the cell appears to be in contact with the visceral musculature at a putative neuromuscular junction. Each lettuce head cell is positioned just below one or two longitudinal muscle strands.

The organization of the SCANS with its connections to the more anterior dorsal caeca is counter to current documented organization of the anterior larval midgut. Just posterior to the proventriculus two sets of gastric caeca, one ventral and another dorsal, project from the intestinal tube. Although poorly documented, the gastric caeca are believed to secrete enzymes and other substances involved in the digestive process (Demerec, 1967). The currently accepted dogma maintains that both sets of gastric caeca project anteriorly (Demerec, 1967), however, I have found that the two dorsal gastric caeca, initially extend anteriorly, but then fold back onto themselves and attach to the midgut at the SCANS region. The attachment seen here is by a specialized group of longitudinal muscles that express high levels of DLG that is located in a novel intracellular bundle. The role of this connection is intriguing and suggests that perhaps the anterior portion of the larval midgut is electrically isolated and functions as an independent unit from the rest of the gut.

Cloning of the *DJ752* Enhancer Trap and What It Reveals about the LHC

For this thesis I have cloned a *Gal 4* enhancer trap, *DJ752*, which is expressed exclusively in the LHC in third instar larvae. In four independently rescued plasmids from genomic DNA extracted from *DJ752* flies I found that the *pGawB* inserted in the *Enhancer-of-Split* Complex [*E(spl)*] in the upstream regulatory region of the *HLHm7* gene. The [*E(spl*)] region in the *Drosophila* genome is the locus at the endpoint of the Notch pathway during embryonic neurogenesis (Jennings, et al., 1994), therefore genes are the target for Notch, and encode repressor proteins (Portin, 2002). The complex

encodes seven related *basic-helix-loop-helix* (*bHLH*) transcription factors which are expressed in response to Notch activation (Jennings et al., 1994). These seven *bHLH* genes are expressed in two neurogenic regions, anterodorsal cephalic and the ventral trunk, during the period when neuroblasts are forming in the embryo. Notch signaling is directly responsible for the accumulation of these proteins in these regions (Jennings et al., 1994). However, Notch signaling is also responsible for continual synthesis of these proteins to temporarily maintain cells in an undifferentiated state so they can later respond to differentiation signals (Jennings et al., 1994). The *E(spl)bHLH* proteins are detected in many other tissues during embryogenesis such as visceral mesoderm and if *E(spl)bHLH* expression is lost they have the potential once more to become neural (Jennings et al.,1994). Therefore, some of the cells that expressed *E*(*spl*)*bHLH* proteins can subsequently re-enter the neural pathway, like enteroendocrine cells embedded within visceral mesoderm epithelial cells (Jennings et al.,1994). The location of the *pGawB* insertion in the *E(spl)* complex certainly suggests that the LHC are in fact, a type of enteroendocrine cell, or a primitive form thereof, since they are an endodermally derived cell type that has neuronal properties. It is likely that these cells lose *E(spl)bHLH* expression, and develop into electrically active cells during embryogenesis, and are embedded within epithelial cells where *E(spl)bHLH* expression has remained active.

Research has revealed that stem cells that span the length of the midgut will develop into pluripotent enteroblasts (EB) that can take on one of two fates (Ohlstien and Spradling, 2006). The stem cell can either develop into an absorptive enterocyte or an enteroendocrine cell. High Notch signaling will block the pathway to the

enteroendocrine cell and the EB will develop into the enterocyte, while low Notch signaling will allow an enteroendocrine cell to form (Ohlstien and Spradling, 2006). It is rather confusing that gene expression dependent upon Notch signaling would be found in LHCs, since low levels of Notch allow for the enteroendocrine cell to develop. Therefore, it is very possible that we have discovered a non-conical differentiation pathway that allows for the LHCs to form and little can be concluded about the development of the LHCs from our cloning results.

The LHC May Be Analogous to Human Cell Types Present in the GI Tract

The LHC have a distinct and interesting morphology. The luminal face of the cell is composed of a highly folded lamellipodia, hence its name. A cap of actin is located just basal to the luminal lamellipodia. Whether these structures of LHC are sensory or excretory remains unknown. There are two types of cells with similar morphology present in the human digestive tract, the Interstitial Cells of Cajal (ICC) and the L-Cells in the duodenum. As mentioned before, the LHC express nervous system markers which indicate that these cells are secreting two types of neurotransmitters, and from our findings we know that the LHC are electrically active and are responsible for regulating peristalsis through the midgut. The ICC are neuron-like cells in the smooth muscle of the human digestive tract, and in the heart, which regulate the dominant pacemaker cells responsible for peristalsis (Sanders and Ward, 2006). They are situated at or between the edges of visceral muscle layers, both circular and longitudinal (Ward and Sanders, 2006), and are close to enteric neurons (Sanders and Ward, 2006). The ICC are essential for a

functional cholinergic excitatory and nitrergic inhibitory motor innervation of the smooth muscle (Ward and Sanders, 2006). Research has revealed that rather than enteric motor nerves innervating smooth muscle cells directly, nerve terminals interact with ICC via synaptic junctions and the innervation occurs via activation of specific receptors and down stream signaling pathways in ICC (Ward and Sanders, 2006). This cholinergic excitatory innervation occurring via these specialized electrically active ICC cells might be exactly what is occurring with the LHC in the midgut of the fly since there is possible Ach secretion from these cells. It is also a convincing argument that the possible DA secretion from the LHC cells, as indicated by expression of the *Ddc Gal 4* line, is part of the same, or a similar, inhibitory pathway as the nitrergic inhibitory innervation seen in ICC. This is evidenced by the long-term treatment and prevention of gastric slow-wave dysrhythmias, where peripheral dopamine receptor antagonist, domperidone, is used to stop ICC inhibition pathways to produce gut emptying (Owyang and Hasler, 2002). DA antagonists are used as treatment for disorders of the bowel which are caused by inappropriate ICC inhibitory function. This suggests that dopamine receptors plays are vital in the inhibitory pathway. If our LHC are primitive forms of the ICC, that would explain the *Ddc Gal 4* expression pattern.

Another enteroendocrine cell type with similar morphology to the LHC, are L-cells. These specialized cells are located in the human duodenum and secrete glucagon-like peptide-1 (GLP-1) in response to the presence of glucose (Jang et al., 2007). Despite the fact that we have shown that the LHC are electrically active cells, their shape and lamellipodial projection more resemble the shape of the L-Cells. These cells have an

apical projection extending into the gut lumen that is highly organized (Jang et al, 2007) and a thicker basal portion with an easily detectable nucleus. The L-cells, much like the taste cells of our tongue, actually "taste the sweetness of glucose" through the G-protein gusductin and other taste transduction elements (Jang et al., 2007). Due to the fact that this apical projection shows a much denser stain, when stained with the ά – subunit of gustductin, it can be concluded that much of the "tasting" occurs here (Jang et al., 2007). The release of GLP-1 as a result of "tasting glucose" in the digestive tract, allows for regulation of appetite, insulin secretion, and gut motility (Jang et al., 2007). It is possible that the highly ruffled lamellipodial ending of LHC is indeed a projection of the cell that tastes for the presence of a chemical compound, and in turn, is influencing an increase in gut motility.

Discussion of Results

Using the *UAS-ricin/Gal4/Gal80* cell ablation technique, we discovered that removal of the LHC from the SCANS region results in a loss of gut motility. SCANS regions lacking LHC have virtually no coordinated muscle contractions. In addition, ectopic activation of these cells using the *CHR2*/blue light techniques (Scholl et al., 2006) shows that the activation of the LHC via exposure to a pulsed blue light results in a muscular contraction within the SCANS region. Furthermore, these results demonstrate that, in addition to the expression of neural genes such as *Ddc* and *Cha*, the LHC are an electrically active cell type and a direct source of contractions within the SCANS region.

With the knowledge that the LHC are electrically active cells that possibly secrete neurotransmitters to influence smooth muscle behavior, the resemblance to the function of the human ICC cells is quite undeniable. However, with a much more similar morphology to the L-Cells in the human duodenum, it is hard to discount that they could be functioning as this type of enteroendocrine cell, given the newly acquired genetic information, and their endodermal origin. It is quite possible that the LHC could be functioning as a primitive form of both these types of cells: tasting the contents of the gut and directly regulating pacemaker activity as a result. Further experimentation is required to determine if in fact, the contents of the food being digested influences gut motility since the results of my feeding assay do not yield an answer to this question.

The results of the ablation contraction experiments further reinforce that anterior portion of the larval midgut, specifically the SCANS, functions as an electrically independent unit. Ablation of the LHC has shown that the midgut peristaltic response does not occur when the LHC have died. The data leading to this conclusion were highly significant when compared to the control groups. The data from the feeding and morphological experiments however, did not have enough significance to lead us to believe that ablated LHC had any affect on digestion or morphology of the SCANS region in the midgut. A diminished amount of feeding in the two hour period was only significantly different in one experimental group out of the four used for the feeding assay. A reasonable explanation for this difference is that the majority of the larvae from the *UAS ricin/+; ChaGal4/ Gal80^{ts}* experimental group died, or became completely unable to move normally because of the death of Cholinergic neurons throughout the

body. It is interesting that we did not see these same results in the other *UAS reaper/+; ChaGal4/ Gal80^{ts}* experimental group. Knowing that our *Gal 4/Gal80^{ts}* system was working, I can only conclude that this result is seen because it takes cells longer to die via programmed cell death (*reaper* – apoptosis) than it does for the potent *ricin* cytotoxin to be rapidly internalized by these cells and kill them (Marsden, et al., 2005). The fact that the loss of only the LHC in the *UAS ricin;reaper/+; DJ752Gal4/ Gal80^{ts}* experimental groups has no effect on the speed in which food is moved through the gut is perplexing. As stated before, contraction data has shown that peristaltic movement used to move food down the alimentary canal is decreased or eliminated when LHC cells have died, and one would think with the disappearance of this motility the ability to pass food properly would be compromised. It is possible that certain factors interfered with the feeding experiment itself. The six trials took place over a number of different days, at different times, with different groups of larvae, and even with food that was not fresh (refrigerated after being made). I think that this experiment needs to be repeated with five to six different groups on the same day, and time, using fresh food, and with a fly progeny that is not aged. I observed that over time it appears that the larvae containing the cytotoxin genotype seem to disappear as egg laying perpetuates over time. The number of "non-glowing" experimental larvae seems to oddly decline in large numbers as the parent flies are transferred each day. Another factor that needs to be controlled further is the amount of time in which cells are allowed to die after being incubated, as making sure the cells containing GFP/Cytotoxin have died, and are not still in the process when placed on white food, is paramount to the success of the study.

Even with all these outside factors being controlled, it is possible that the speed in which normal agarose gel food moves through the gut will not be changed with the disappearance of the LHC. In which case, further experimentation with different types of food, containing different amounts of yeasts, sugar, and even caffeine will yield some insight into how the loss of the LHC actually affects the digestive process.

The results from the second LHC ablation assay, showed that overall, the morphology of the midgut does not change when these cells die. In every comparison, we saw no difference across groups in overall diameter of the SCANS region or luminal diameter. There were some slight decreases in gut wall diameter when the experimental groups were compared to the external wild-type control groups, but this significant data did not hold true when experimental groups were compared to the internal wild-type controls. Even though this result seems odd, a factor that might be assisting in the difference of gut wall diameter between groups is the condition in which the larvae are being treated. The external wild-type controls were not placed at 29°C overnight, whereas, the internal wild-type controls were incubated overnight along with experimental groups. The pattern of results suggests that there is a morphological change, in gut wall diameter, happening because of the temperature conditions rather than because LHC are being ablated. The percentage of open lumens tabulated also followed this trend. To eliminate this variable, all that is needed are gut wall measurements of a group of external wild-type control larvae after overnight incubation.

External variables were also a factor in the *Channelrhodopsin-2* gain of function experiments. Data was highly significant for the experimental groups when larvae

exposed to blue light first were compared to the control groups exposed to white light first. In the case of *DJ752*, exposure to blue light resulted in a highly significant increase in contractions compared to *DJ752* and W^{1118} exposed to white light first, as well as W^{1118} exposed to blue light. Although the results used exposure to either blue or white light first, all larvae used in the experiments were exposed to both types of light consecutively. The data for blue light exposure second, appeared to be odd when compared to the data for blue light exposure first, and such was the case for both types of experimental situations. My own observations of dissecting larvae under white light that remained in solution for an extended period of time, leads me to believe that the retinal compound actually leaks out of the specimen and there may be some type of bleaching occurring under high levels of white light. Therefore, when the larval gut specimen was exposed to the high level of white light prior to being exposed to blue light, photobleaching and/ or leaking of retinal could have been occurring. My data show that blue light specimen's, especially in the case of *DJ752*, contractions were less in number than the white light contractions seen right before blue light exposure, and less than the wild-type example. This result seems extremely contradictory of the results examined in Table 5, and would have been a strong indicator that the LHC are not electrically active, if the same type of trend was not seen in the opposite case. When larvae exposed to blue light first were compared to white light second, the same type of decrease in contractions was observed. This, of course, in both experimental groups made blue light data vs. white light data extremely significant, so again another outlying variable was observed. Therefore, to eliminate these variables, only white light first and blue light first data were compared.

Future Experiments and the Big Picture

Peristaltic contraction data in both ablation and ectopic activation experiments strongly suggested that the LHC are required for stimulating and perpetuating electrical muscle contractions throughout the midgut of the larval intestine. Future experiments are needed to further characterize the function and physiology of the LHC. Some questions include; why they exhibit the morphology they have, if the loss of the LHC effects development, and if they are in fact sensitive to certain types of chemical compounds in food. Overall, the most important and fundamental step needed to perpetuate the study of these cells is to create a viable mutant that lacks them. With a mutant, the variables that caused issues in the ablation experiments will be eliminated; such as the concern that the cytotoxin is specifically eliminating the LHCs and not penetrating other cells.

The big picture in pursuing the function of the LHC in the SCANS region of the midgut is to take a step towards having an easily obtainable and easily manipulated model organism to study the mammalian gut. The number of diseases and disorders of the intestine that effect the lives of people every day is so enormous it is no wonder that the abdomen is referred to as the "black box" of internal medicine. The loss of gut motility resulting from the death of the LHC in the midgut of the fly relates to a number of diseases seen when the ICC-MY of the small intestine are absent or stop functioning. Experiments involving mice where the c-Kit pathway responsible for the development of ICC-MY was blocked, showed a loss of pacemaker activity and a loss of the ICC network in the intestine, which ultimately results in a loss of gut motility (Burns, 2007). This disruption of electrical activity is reported in a wide variety of gastrointestinal diseases

where motility of the gut has completely ceased or malfunctioned in some sort of way (Burns, 2007). These diseases include, but are not limited to: achalasia, chronic intestinal pseudoobstruction, Hirschsprung disease, inflammatory bowel diseases, slowtransit constipation, ulcerative colitis, and Crohn's disease (Burns, 2007). If, in fact, the LHC in the midgut of the fly are a homologue to the ICC seen in the human intestine, or even the ICC present in the human heart, the larval intestine will prove to be a very accessible model to study and develop treatment for the diseases outlined above and many others. Although there are already mammalian models being used, like mice, the *Drosophila* model has time on its side. While mice have a gestational period of about 20 days, fruit flies can be mated and have larvae ready to be experimented on in under a week. *Drosophila* are easily obtainable and easy to work with genetically, so having a model like this available certainly wouldn't replace mammalian studies, but merely add to them.

Conclusion

The discovery of this novel, electrically active cell type within a new region of the *Drosophila* larval midgut may prove to be a new model system in which gut and cardiac electrical pacemaking functions can be studied. This novel region in the anterior midgut is known as the Superior Cuprophilic Autonomic Nervous System (SCANS). Within this region a novel cell type, the Lettuce Head Cells (LHC), have been discovered and the anatomical placement of the dorsal gastric caeca has been re-arranged. Several enhancer traps have been shown to label the larval midgut, but a few exclusively express in the LHC. *DJ752* is an intriguing one because it only expresses in the LHC during third instar

larvae. This *Gal 4* line was cloned because discovering what genes are involved in the development of the LHC aids us in understanding its function as an electrically active enteroendocrine cell. Loss of the LHC results in extreme reduction, if not loss, of peristalsis throughout the anterior midgut. Ectopic activation of these cells shows that gut contractions are increased when the LHC are electrically excited. Therefore, I have been able to conclude, through these series of experiments, that the LHC are required for larval midgut peristalsis.

REFERENCES

Ameil J., Sproat-Emison E., Garcia-Barcelo M., Lantieri F., Burzynski G., Borrego S., Pelet A., Arnold S., Miao X., Griseri P., Brooks A.S., Antinolo G., de Pontual L., Clement-Ziza M., Munnich A., Kashuk C., West K., Wong K.K., Lyonnet S., Chakravarti A., Tam P.K., Ceccherini I., Hofstra R.M., Fernandez R.. (2007). Hirchsprung disease, associated syndromes and genetics: a review. *American Journal of Medical Genetics* 45(1):1-14.

Baylies K., Bate M., Ruiz Gomez M. (1998). Myogenesis: a view from *Drosophila. Cell* 93: 921-7.

Benoit S., Tracy A. (2008). Behavioral controls of food intake. *Peptides* 29:139-147.

Bodenstein, D. (1950). The postembryonic development of *Drosophila*. Biology of Drosophila (ed. M. Demerec), pp. 275-367. New York: John Wiley and Sons.

Brand A., Perrimon N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401-415.

Budnik V., Wu C.F., White K. (1989). Altered branching of serotonin-containing neurons in *Drosophila* mutants unable to synthesize serotonin and dopamine. *Neurosci* 9(8): 2866-77.

Burns A. (2007). Disorders of Interstitial Cells of Cajal. *Journal of Pediatric Gastroenterology and Nutrition* 45: S103-S106.

Chaudhri O., Small C., Bloom S. (2006). Gastrointestinal hormones regulating appetite. *Philosophical transactions of the Royal Society of London.* Series B: Biological sciences 361(1471):1187-209.

Cheng H., Leblond C. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. *Am. J. Anatomy* 141: 537-561.

Demerec, M. (Ed.). (1965). Biology of Drosophila. New York, NY: Hafner Publishing Company, Inc.

Dubreuil R.R. (2003). Copper cells and stomach acid secretion in the *Drosophila* midgut. *The International Journal of Biochemistry & Cell Biology* 36:745-752.

Dubreuil R.R., Frankel J., Wang P., Howrylak J., Kappil M., Grushko T.A. (1998). Mutations of a spectrin and labial block Cuprophilic Cell differentiation and Acid Secretion in the Middle Midgut of *Drosophila* larvae. *Developmental Biology* 194: 1-11.

Duffy J.B. (2002). *Gal 4* system in *Drosophila*: A fly geneticist's Swiss army knife. *Genesis* 34: 1-15.

Evers B. M. (1999). Intestinal Cell Differentiation: Cellular Mechanisms and the Search for the Perfect Model Focus on "Involvement of p21 (WAF1/Cip1) and p27 (Kip1) in intestinal epithelial cell differentiation." *Am J Physiol Cell Physiol* 276: C1243-C1244.

Goldstein M.A., Burdette W.J. (1971). Striated visceral muscle of *Drosophila melanogaster*. *J Morphol.* 134(3): 315-34.

Hidalgo A., Urban J., Brand A.G. (1995). Targeted ablation of glia disrupts axon tract formation in the *Drosophila* CNS. *Development* 121: 3703-3712.

Hrdlicka L., Gibson M., Kiger A., Michelli C., Schober M., Schock F., Perrimon N. (2002). Analysis of Twenty-Four Gal4 lines in *Drosophila* melanogaster. *Genesis* 24: 51-57.

Jang H., Kokrashvili Z., Theodorakis, M., Carlson O., Kim B., Zhou J., Kim H., Xu X., Chan S., Juhaszova M., Bernier M., Mosinger B., Margolskee R., Egan J. (2007). Gut-expressed gustducin and taste receptors regulate secretion of glucagons-like peptide-1. *PNAS* 104(38):15069-74.

Jennings B., Preiss A., Delidakis C., Bray S. (1994). The Notch signalling pathway is required for *Enhancer of split* bHLH protein expression during neurogenesis in the *Drosophila* embryo. *Development* 120: 3537-3548.

Klapper R., Stute C., Schomaker O., Strasser T., Janning W., Renkawitz-Pohl R., Holz A. (2002). The formation of syncytia within the visceral musculature of the *Drosophila* midgut is dependent on *duf, sns* and *mbc*. *Mech of Dev* 110(1-2): 85-96.

Klapper R., (2000). The longitudinal visceral musculature of *Drosophila melanogaster* persists through metamorphosis. *Mechanisms of Development* 95: 47-54.

Kusch T., Reuter R. (1999). Functions for *Drosophila brachyenteron* and *forkhead* in mesoderm specification and cell signaling. *Development* 126: 3991-4003.

Lee C., Cooksey B., Baehrecke E. (2002). Steroid regulation of midgut cell death during *Drosophila* development. *Developmental Biology* 250: 101-111.

Marsden C.J., Smith D.C., Roberts L.M., Lord J.M. (2005). Ricin: current understanding and prospects for an antiricin vaccine. *Expert review of vaccines* 4(2): 229-37.

Meyer J.S., & Quenzer L.F. (2005). Psychopharmacology. Sunderland, MA: Sinauer Associates, Inc.

Micchelli C.A., Perrimon N. (2006). Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature* 439(7075): 475-9.

Murakami R., Takashima S., & Hamaguchi T. (1999). Developmental genetics of the Drosophila gut: specification of primordia, subdivision and overt-differentiation. *Cellular and molecular biology*. *45(5)*: 661-76.

Nakagoshi H. (2005). Functional Specification in the *Drosophila* endoderm. *Developmental Growth and Differentiation* 47: 383-392.

Ohlstein B., Spralding A. (2006). The adult *Drosophila* posterior midgut is maintained by pluripotent stem cells. *Nature* 439(7075): 470-4.

Owyang C., Hasler W. (2002). Physiology and Pathophysiology of the Interstitial Cells of Cajal: From Bench To Bedside VI. Pathogenesis and therapeutic approaches to human gastric dysrhythmias. *Am J Physiol Gastrointest Liver Physiol* 283(1): G8-G15.

Park S.S., Shahabuddin M. (2000). Structural organization of posterior midgut muscles in mosquitoes, *Aedes aegypti* and *Anopheles gambiae*. *Journal of Structural Biology* 129(1): 30-7.

Portin P. (2002). General outlines of the molecular genetics of the Notch signalling pathway in Drosophila melanogaster: a review. *Hereditas* 136(2):89-96.

Ranganayakulu G., Elliott D.A., Harvey R.P., Olson E.N. (1998). Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. *Development* 125: 3037-3048.

Sanders K., Ward S. (2006). Interstitial cells of Cajal: a new perspective on smooth muscle function. *J. Physiol.* 576(3): 721-26.

Sbarbati A., Osculati F. (2007). Extending the enteric nervous system. *Biomedicine & Pharmacotherapy* 61: 377-382.

Scholl C., Riemensperger T., Bucher D., Ehmer J., Voller T., Erbguth K., Gerber B., Hendel T., Nagel G., Buchner E., Fiala A. (2006). Light-Induced Activation of Distinct modulatory Neurons Triggers Appetitive or Aversive learning in *Drosophila* Larvae. *Current Biology* 16: 1741-1747.

Seroud L., Brummel T., Kapahi P,Benzer S. (2002). Spatio-temperola analysis of gene expression during aging in *Drosophila melanogaster*. *Aging cell* 1: 47-56.

Shimizu Y., Matsuyama H., Shiina T., Takewaki T., Furness J. B. (2008). Tachykinins and their functions in the gastrointestinal tract. Cell. *Mol. Life Sci.* 65: 295 – 311.

Suster M.L., Seugnet L., Bate M., Sokolowski M.B. (2004). Refining GAL4-driven transgene expression in Drosophila with a GAL80 enhancer-trap. *Genesis.* 39(4): 240-5.

Valassi E., Scacchi M., Cavagnini F. (2008). Neuroendocrine control of food intake. *Nutrition, Metabolism & Cardiovascular Diseases* 18(2):158-68.

Ward S., Sanders. K. (2006). Involvement of intramuscular interstitial cells of Cajal in neuroeffector transmission in the gastrointestinal tract. *J Physiol* 576(3): 675-682.

Wood J. (2008). Enteric nervous system: reflexes, pattern generators and motility. *Current Opnion in Gastroenterology* 24:149-158.

Zeidler M.P., Tan C., Bellaiche Y., Cherry S., Häder S., Gayko U., Perrimon N. (2004). Temperature-sensitive control of protein activity by conditionally splicing inteins. *Nat Biotechnol* 22(7): 871-6.